

STATE LIBRARY OF PENNSYLVANIA



3 0144 00328607 7

S

614.405

5826

V.35





Digitized by the Internet Archive
in 2015

<https://archive.org/details/journalofinfecti35unse>

The
Journal of Infectious Diseases

Published by the John McCormick Institute for Infectious Diseases

EDITED BY
LUDVIG HEKTOEN AND EDWIN O. JORDAN

IN CONJUNCTION WITH
FRANK BILLINGS F. G. NOVY
H. GIDEON WELLS KARL F. MEYER

Volume 35
1924

Chicago, 1924

Composed and Printed by
American Medical Association Press
Chicago, Illinois, U. S. A.

NEUMOC

mo

STABILITY OF PNEUMOCOCCUS TYPES IN STERILE ABSCESES

EMERSON MEGRAIL AND E. E. ECKER

From the Departments of Hygiene and Bacteriology, and of Pathology, School of Medicine, Western Reserve University, Cleveland, Ohio

We have shown¹ that relative inagglutinability of *Bact. typhosum* and of *B. pestis-caviae* could be induced by growing these organisms in sterile gum tragacanth abscesses in mice.

Experiments by one of us (Ecker) demonstrated that relative inagglutinability of *Bact. typhosum* could also be induced by growth in 20% sterile normal human serum broth (6 transfers), and that the serum of the atypical typhoid patient described by Blankenhorn, Ecker and King² produced a similar alteration of a normal strain to the same degree. The strain isolated in this case, however, remained inagglutinable under the same conditions and was not made more agglutinable by growth in convalescent typhoid serum, while the ordinary laboratory strain sedimented out in dilutions from 1:80-5,000 in fine floccules following 6 transfers in this serum broth.

Because of varying results obtained by others, we thought it advisable to study pneumococci in fixation abscesses.

Changes of agglutinability have been produced by Porges,³ who made the poorly agglutinable *Streptococcus mucosus* more agglutinable by treatment with HCl. Similar experiments were made by Hanes,⁴ and by Nicolle, Juan and DéCains,³ who concluded that group 4 organisms could be eliminated by bringing about agglutinability. According to Nicolle, normal serum agglutinates the majority of pneumococci examined, but not all are thus affected by immune serum. Colonies isolated from blood were found to be agglutinated by 2 serums, while a perirenal abscess strain failed to be agglutinated. Friel⁵ found that by growing pneumococci in immune serum the organism became agglutinable in normal rabbit serum. Stryker⁶ pointed out that growth in immune serum lessened agglutinability in immune homologous serum, and caused agglutina-

Received for publication, March 31, 1924.

¹ Jour. Infect. Dis., 1923, 33, p. 269.

² Ibid., 1923, 32, p. 95.

³ Quoted by Cotoni, Truche and Raphael: Monograph, Pasteur Institute, 1922.

⁴ Jour. Exper. Med., 1912, 16, p. 512.

⁵ Publication So. African Inst. Med. Res., Jan. 26, 1915.

⁶ Jour. Exper. Med., 1916, 24, p. 49.

tion in heterologous and even normal serums. Blake and ⁷ also reported changes when grown in homologous immune serum. Adler,⁸ on the other hand, was unable by ordinary animal passage to change type 4 strains to other types. Felton and Dougherty⁹ have shown that after attenuation of a known fixed type 1 pneumococcus in broth the organism regained virulence and marked increase of agglutinability in milk and was also agglutinated in heterologous serum.

TECHNIC

Essentially the same methods were used in this work as before.¹ Approximately 0.5 c.c. of a sterile 2% gum tragacanth suspension was injected subcutaneously into a mouse and from 1 to 2 c.c. into the rat. From 0.2 to 0.4 c.c. of a suspension in salt solution from a blood agar slant of the organism was injected into this abscess. In the first set of experiments there was an interval of 24 hours between the injection of the gum tragacanth and the organisms in the first 8 transfers in the animal. In the rest of this series and in all later experiments the organisms were placed in the abscess 6 hours after its formation. This change was made in an attempt to get the organisms in contact with leukocytes soon after migration and before disintegration. The organisms were not withdrawn from the abscess until the animal showed signs of being in a serious condition. The abscess material was stained, studied and plated. Several colonies were isolated from each plate and the mixed strains inoculated into the next animal. This was done to exclude variants. Agglutination tests were made with bacterial suspensions of the same density by placing one drop of the suspension and one drop of the serum dilution in the concavity of a hanging drop slide, placed in a moist chamber in a water bath at 37 C. Examinations were made macroscopically and microscopically at the end of one and two hours.

Tentative experiments in which a recently isolated type 2 culture was passed through abscesses in 11 mice showed that the strain agglutinated in a dilution of 1:10 of homologous serum, while the original agglutinated +++ at a dilution of 1:40 of the serum. The virulence of the organism decreased as the first two animals died within 48 hours of injection, but the last 9 survived. The growth of the transferred strain was similar to that described by Stryker after growth in immune serum. The culture on blood agar was dry and brownish. Hemolysis was observed on plates of old cultures.

From these results it was then thought advisable to use fixed strains. Cultures from the New York State Board of Health, obtained through the kindness of Dr. A. B. Wadsworth, were used in the first series of experiments. Of these cultures, 5, type 1, was passed through abscesses in 15 mice; 122, type 3, was passed through 16 mice, and D 5 C., type 2, was passed through 6 white rats. No change in bile solubility or inulin fermentation was produced in the passed strains. In no case was it possible to keep the culture in the animal for more than 24 hours. In the direct smears from the abscesses little evidence of phagocytosis was observed and the impression was given that the polymorphonuclear leukocytes had been practically destroyed while the mononuclear leuko-

⁷ Abst. Bacteriol., 1923, 7, p. 353.

⁸ Ztschr. f. Hyg. u. Immunitätsf., 1923, 101, p. 140.

⁹ Jour. Exper. Med., 1924, 39, p. 155.

cytes remained morphologically intact. Agglutination tests were made comparing the passed strains with the original strains in homologous serums. The animal passed strains all showed clumping at the same titer as the original cultures. In cross agglutination tests the animal passed strains showed no tendency to clump in other type serums.

Three type 4 cultures, obtained through the courtesy of Dr. Charles Krumwiede, were each passed through 7 mice in the same way. The animals with these cultures lived on the average some hours longer, but for the most part it was necessary to withdraw material from the abscess after the organisms had been left in for 24 hours. Here again there was little evidence of phagocytosis, and there was the same picture of disintegrated polymorphonuclear leukocytes. Agglutination tests showed clumping in undiluted and 1:15 type 2 serum of all original and passed strains but no agglutination in higher dilutions of this serum or of the other serums.

Intraperitoneal injections were made using 0.5 c.c. of type 1 agglutinating serum in one series of mice, type 2 serum in another series and type 3 serum in a third series, each series comprising 8 mice. Type 4 cultures were then injected into preformed abscesses. Agglutination reactions of these transferred cultures showed no change.

In an attempt to keep the organisms in contact with abscess contents for a longer period, type 1 therapeutic serum was injected in 0.5 c.c. amounts with 0.2 c.c. of a suspension of type 1 organisms into abscesses in 3 successive mice. The organisms were withdrawn after 2 days and introduced into the next mouse, giving a total of 6 days' contact between organism and abscess contents. Agglutination reactions of the culture thus transferred showed no change from the original. There was in this case, however, much less destruction of the polymorphonuclear leukocytes, and phagocytosis was more pronounced than in any of the preceding experiments. The mice in each case died in less than 72 hours.

DISCUSSION AND SUMMARY

It is evident from these experiments that the tissues or fluids in a sterile fixation abscess do not in the case of the pneumococcus contribute toward alteration of agglutinability as was the case with *Bact. typhosum* and *B. pestis-caviae*. In both of these cases animals were used which were susceptible and resistant to the organisms. In both cases morphologic and cultural characteristics were unchanged by this method of animal passage. Type 1 organisms in contact with type 1 serum and abscess contents showed the same resistance to this immunologic change.

Type 4 organisms, which with our serums were called subtype 2 cultures, were likewise unchanged. This was also true when the animals had received injections of agglutinating serums of the other 3 types. It was also apparent that there was destruction of polymorphonuclear leukocytes in the abscess, while the other white cells showed little damage.

Considering the recent work of Felton and Dougherty,⁹ we must assume that variations occur since daughter cells show great variability, possibly explaining conflicting results of other workers. We have used only cultures made from several colonies prior to each transfer.

From our observations it may be concluded that the pneumococcus has a type stability when placed under conditions in which typhoid bacilli and also other organisms show variability in agglutination.

SURFACE ENERGY AS THE CONTROLLING FACTOR IN AGGLUTINATION AND DISPERSION

R. G. GREEN AND H. O. HALVORSON

*From the Department of Bacteriology and Immunology University of Minnesota,
Minneapolis, Minn.*

When a uniform and stable suspension of bacteria changes into a flocculent mass of clumped cells, the change in state is so evident that it long ago stimulated interest in the forces active in bringing about the transformation. The first definite clue as to the nature of these forces came from the observation of Bechold¹ that bacteria as ordinarily grown move to the anode when in a potential gradient. Bechold established also that heated bacteria exhibited this property as well as the live cells, and his observations have been confirmed by many later investigators. This movement in an electric field has been interpreted as meaning that the bacterial cells have a charge, and it has been assumed that this charge is of such a nature that bacterial cells having this charge will repel each other, and this force of repulsion is active in maintaining a stable suspension. However, the work of Bechold,¹ Teague and Buxton,² and Arkwright³ has established that bacteria exhibit the evidence of this charge even when in an agglutinated state. Recently Northrop and De Kruif⁴ have demonstrated, that the charge may be reduced and definitely reversed, and they found a definite relation of these changes to the occurrence of agglutination. Since the charge is not necessarily absent when agglutination takes place, it has been evident to some investigators that another force must be present in the system which tends to bring the bacteria together on agglutination, and that this force must be greater in magnitude than the force due to the charge remaining on bacteria when agglutinated. Buchanan⁵ referred this force to that of surface tension and assumed that this force acted oppositely to that exerted by the electric charges. To quote Buchanan's description of the phenomenon: "Agglutination occurs whenever the similar electric charges are decreased to amounts such that they will no longer overcome

Received for publication, April 10, 1924.

¹ Ztschr. f. physiol. Chem., 1904, 48, p. 385.

² Ibid., 1907, 57, p. 76.

³ Jour. Hyg., 1914, 14, p. 261.

⁴ Jour. Gen. Physiol., 1922, 4, p. 639.

⁵ Jour. Bacteriol., 1919, 4, p. 73.

the pull of surface tension, or conversely, surface tension may be increased until it overcomes the dispersion effect of the similar charges."

Northrop and De Kruif carried out their experimental work on the basis of the foregoing hypothesis, measuring changes in the electric potential between the cells and the medium by rate of movement in an electric field and the changes in the "cohesive force" by pulling apart glass slides covered with a film of bacteria. Northrop and De Kruif found that whenever the potential difference between the surface of the bacteria and solution was less than about 15 millivolts, the bacteria agglutinated, provided the cohesive force was not affected. It was further found that if the cohesive force was less, the critical potential decreased. And, still more important as regards our consideration, they also found that if the cohesive force was small, no agglutination took place even though the potential was zero. The term "cohesive force" used by these investigators was not defined, but they suggested that surface tension was probably a factor, as was indicated by Buchanan.

It is our purpose to point out the distribution of free energies which are present in such a system as a bacterial suspension and to show how various changes in the free energy distribution will occur, and so determine whether the cells will exist evenly distributed or agglutinate into clumps. In the following, the energy terms will all refer to free energies.

We may assume at once that there are three groups of free energies, that associated with the masses of the phases, the electrical energies, and the energies associated with the interfaces. In this discussion, we will consider the mass energies constant. When changes in state occur, we will consider the change as occurring after the mass of any substance added has become a part of the system and direct our attention to the subsequent change in state, the actual dispersion or agglutination. The effect of the charge of bacteria will not enter into this consideration to any great extent. It is present before and after agglutination under ordinary conditions. When the charge is absent, agglutination may or may not occur. This indicates that other forces are of primary importance. Further, we may say that cataphoresis indicates that a particle is charged with respect to the medium with which it is in contact. Such a charged particle is surrounded by a layer oppositely charged. We do not feel that the charge attributed to bacterial cells has been shown to be of such a nature that it exerts any appreciable repulsive effect toward any other similarly charged bacterial cells. Again, we may consider that in agglutination the outer surface zone of two bacteria may be in contact

and a plausible assumption for such a situation would be that being similar there would be no potential difference at the cell-cell interface.

This leaves us but the interfacial energies in which to look for the forces primarily responsible for the change in state observed in agglutination. It is to be pointed out that we are dealing with small masses where the ratio of surface to volume is very high and that in such systems in which the specific surface is very large, the surface energy is a large part of the total energy of the system. It is not convenient to follow the custom of considering the forces concerned with agglutination. To do so, we would consider the surface tension of the various interfaces. Surface tension can be considered a factor in that its value for any interface in dynes per cm. gives us the value of the surface energy in ergs per cm.² We will use the idea of surface energy that we may evaluate the total energy.

It is now necessary that we consider the nature of the interfaces with which we are dealing. We will consider that there is a cell surface of definite thickness which is quite independent of the medium, a cell surface which would exist if the cell were in a medium other than water. We will consider that when the cell is put in a watery solution this cell surface will be built up by a definite thickness by the water surface in contact with it. Into this surface will be adsorbed constituents of the medium, forming what we may speak of as an ectozone. The composition of this zone will depend on the medium. If there is present in the medium a constituent which will reduce the energy of the zone by concentrating there, it will be adsorbed. We will direct our attention specifically to that type of agglutination which we can think of as being brought about by the adsorption of substances into interfaces and thereby changing the energy relationships of the surfaces and the phases. It is to be pointed out that the assumptions as to the interfaces above do not determine the subsequent treatment. The assumptions are made in accordance with what is to be expected from our present ideas of physical and colloidal chemistry, but are definitely set forth for the convenience of explanation.

Let us consider a suspension of bacteria in which some bacteria are in contact as pictured in fig. 1, which we will take to represent a state that is not a stable suspension, nor a state of agglutination, but a state between the two. We may now attempt to define the distribution of surface energies in such a state and also changes in surface energy that will tend to make the suspension agglutinate into clumps or become stabilized so that no cells will be in contact.

In the state as represented in fig. 1, we will consider that there are two interfaces, the interface formed by the cell and liquid, designated by S , and the interface formed when two cells come in contact, designated by S_1 . The interface S is of appreciable thickness and, while complex in composition, it can be treated in a simple manner. We will consider it to be a double zone, which is built up by surface concentration of substances from the solution in its outer part and by surface concentration of cellular elements in its inner part. To the outer part we will assign the average specific energy value e , and to its inner part the average specific energy value e_2 . The cell-cell interface will be formed when two cells come in contact with the inner part of the foregoing zone of two cells in contact and with no solution as such interposed. This zone will then also have a specific energy value e_2 . Now we can say in general

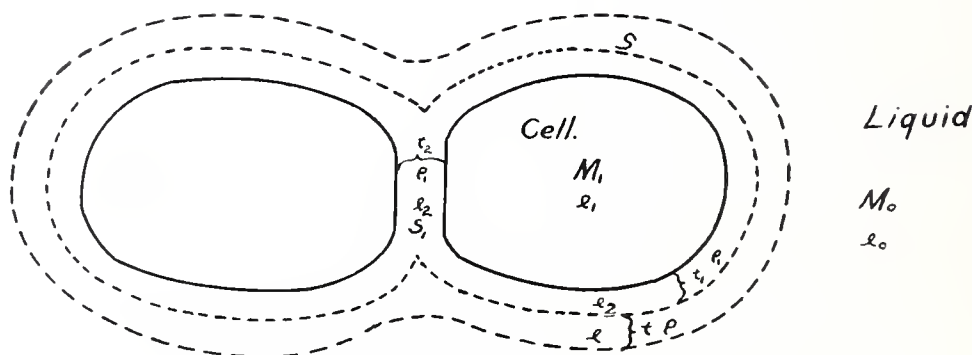


Fig. 1.—Bacterial cells in a state of agglutination.

that if the suspension goes to a completely dispersed state, the interface S will increase to a maximum, and the interface S_1 will disappear. If the suspension goes to a state of agglutination, the interface S will decrease to a minimum and the interface S_1 will increase to a maximum. A ratio of S to S_1 is then a means of indicating the size of agglomeration in any state considered. The various parts depicted in fig. 1 are defined as follows.

M_0 indicates total mass of solution suspending cells; M_1 , total mass of cells; S , area of cell-liquid interface; S_1 area of cell-cell interface; t and ρ , thickness and density, respectively, at any point of the liquid surface in contact with the cell, i. e., the outer zone of the cell-liquid interface; e , specific energy of surface zone $s \approx \rho t$; t_1 and ρ_1 , thickness and density, respectively, at any point of the cell surface, i. e., the inner zone of the cell-liquid interface; t_2 and ρ_1 , thickness and density, respectively, at any point of the cell-cell interface; e_2 , specific energy of cell surface and therefore zone $s \approx \rho_1 t_1$ and zone $s_1 \approx \rho_1 t_2$; e_0 specific energy of solution mass; e_1 specific energy of cell mass; E , total free energy of the system.

We can equate the total energy in terms of the various components, and arrive at the following equation:

$$E = (M_0 - S\Sigma t\rho) e_0 + S\Sigma t\rho e + S\Sigma t_1\rho_1 e_2 + (M_1 - S\Sigma t_1\rho_1 - S_1\Sigma t_2\rho_1) e_1 + S_1\Sigma t_2\rho_1 e_2$$

$$E = M_0 e_0 + M_1 e_1 + S [\Sigma t\rho (e - e_0) + \Sigma t_1\rho_1 (e_2 - e_1)] + S_1\Sigma t_2\rho_1 (e_2 - e_1)$$

Since our system is made up of small bodies, this surface energy becomes highly important, and since we are considering the effect of surface energy on the state of equilibrium, we will assume that the specific energies of the masses are constant. We may therefore write:

$$E = M_E + S [\Sigma t\rho (e - e_0) + \Sigma t_1\rho_1 (e_2 - e_1)] + S_1\Sigma t_2\rho_1 (e_2 - e_1)$$

This equation states that the total energy is composed of two parts, one part constant with the total mass, and the other part varying with the masses associated with the interfaces. A change in the total energy of such a system will vary with the area of the interfaces, and with their thickness and density. The total energy will also vary with those factors that represent the differences of the specific energies of the surface zones and the masses. These factors are of prime importance, as a decrease in any factor may be brought about by surface concentration of surface tension depressants or adsorption. This will give rise to a new distribution of surface energies which may make the system unstable and may bring about a change in the state of the dispersion.

The state pictured in fig. 1 may be an unstable one. Let us consider in what direction a change will occur to make the system more stable. Under what conditions will the system go to a state of more complete dispersion, and under what conditions will the system go to a state of more complete agglutination?

In whatever direction the change is going to occur, there are certain conditions that must be fulfilled. From the laws of thermodynamics, we know that in any spontaneous process at constant temperature and volume, the free energy of the system decreases. From the nature of our system we also know that when S increases, S_1 will decrease, or when S_1 increases, S will decrease. We can, therefore, state that $\Delta S = -\frac{1}{2}\Delta S_1$. There may, of course, be changes possible in which this is not necessarily true. It may be possible to increase both S and S_1 at the same time, as in a case of growth. Also it might be possible to decrease both S and S_1 at the same time, as in the case of lysis. These considerations, therefore, do not come within the scope of this paper, but will be considered in a later publication. We are here concerned with agglutination or dispersion, and we will, therefore, assume that there is no change in shape or volume of cells. The foregoing statement that $\Delta S = -\frac{1}{2}\Delta S_1$ must therefore hold.

By differentiating Eq. 1, assuming that the total energy E and areas S and S_1 are the only variables, we get

$$\begin{aligned} dE = dS [\Sigma t \rho (e - e_0) + \Sigma t_1 \rho_1 (e_2 - e_1)] + dS_1 \Sigma t_2 \rho_1 (e_2 - e_1) = \\ dS [\Sigma t \rho (e - e_0) + \Sigma t_1 \rho_1 (e_2 - e_1)] - 2dS \Sigma t_2 \rho_1 (e_2 - e_1) \\ - dE = dS [(2\Sigma t_2 \rho_1 - \Sigma t_1 \rho_1) (e_2 - e_1) - \Sigma t \rho (e - e_0)] \end{aligned}$$

Since we are not concerned in this paper with the values of the thicknesses and densities, we may write the following equation:

$$-dE = dS [k_2 (e_2 - e_1) - k_1 (e - e_0)]$$

Since in any spontaneous process dE must be negative, we can draw some definite conclusions.

If $k_2(e_2 - e_1) > k_1(e - e_0)$, the coefficient of dS will be positive and the free energy will decrease with an increase of S . This must be the prevailing condition in our system if it is to go to a state of more complete dispersion.

If $k_2(e_2 - e_1) < k_1(e - e_0)$, the coefficient of dS will be negative and the free energy will decrease with a decrease of S . This must be the prevailing condition in our system if it is to go to a state of more marked agglomeration.

At equilibrium $\frac{dE}{dS} = 0$. Therefore, at equilibrium $k_2(e_2 - e_1) = k_1(e - e_0)$. Let us suppose we have a system that is in equilibrium, and then see what the conditions must be to change the system to a new state of equilibrium. Since we are interested in the effect of surface energies on the state of equilibrium, we shall assume that e and e_2 are the only variables and see what effect changes in them will have on the state of equilibrium. How must their values change to change the state of equilibrium to a state of more complete dispersion? From the foregoing deductions, we can see that in order to do this we must either increase e_2 or decrease e . It can also be seen that in order to change our state of equilibrium to a state of more marked agglutination, we must either increase e or decrease e_2 . The effect of substances that increase the surface energies, such as electrolytes, will be considered in a future discussion. We will confine ourselves in this paper to consideration of surface energy depressants, or substances which affect the interfaces by adsorption. We can then state, substances that depress the surface energy of the liquid in the liquid cell interface favor dispersion; and substances that lower the surface energy of the cell favor agglutination. By inspection of the equations, it can be seen that the foregoing deductions hold if either $(e_2 - e_1)$ or $(e - e_0)$ are positive or negative, or if they are both positive or negative. It may be of importance to

discuss these possibilities. If we assume that $(e_2 - e_1)$ or $(e - e_0)$ are positive, we are assuming that the specific surface energy is greater than the specific mass energy. This, of course, is the condition prevailing in a liquid gas interface and is a possible condition. If we assume the negative values, we are assuming that the specific surface energy can be less than the specific mass energy. In this case the total energy would decrease with an increase in surface, and the surface would spontaneously increase.

We see phenomena that resemble such a system, an example of which is the spontaneous rise of water in a capillary tube. When reference is made to the force necessary to increase such a surface (which force would be opposite in sign to that described as positive surface tension), the term "negative surface tension" is used. This term has been vigorously objected to by some authors. It follows that a surface exhibiting negative surface tension has a greater density than the internal mass of the corresponding phase. It also follows that when water is in contact with glass, the pressure on the glass will be as great as or greater than the internal pressure of the liquid. The internal pressure of a liquid, as indicated by the kinetic theory, is very large, and it does not seem tenable that the pressure on the glass can be of so great a magnitude. This may be a real argument against the existence of negative surface tension, but the authors believe this to be the result of our inability to explain completely by the kinetic theory the properties of liquids. The assumption then that $e < e_0$, and $e_2 < e_1$ may then have some objections.

Let us examine again the equation $-dE = dS[k_2(e_2 - e_1) - k_1(e - e_0)]$. If we imagine this process to occur at constant temperature and volume, the change in free energy is a measure of the affinity of the process. The tendency of the process, therefore, to go toward a stable suspension depends on how much larger $k_2(e_2 - e_1)$ is than $k_1(e - e_0)$. If $(e - e_0)$ can be negative, and $(e_2 - e_1)$ is positive, the tendency or affinity will be very large. This, of course, is to be expected.

The foregoing deduction would appear to establish that the distribution of surface energy is the fundamental factor in bacterial agglutination or the stability of bacterial suspensions. It demonstrates that if the free energy of such a system decreases, as it must in any spontaneous process, the result may be agglutination or dispersion. If a bacterial suspension is in a stable state, its free energy is a minimum, and it will remain stable until some new factor is added to the system that will bring about a new distribution of energy whereby the free energy may

be decreased by a change in the ratio of the interfaces. That there is some force holding the cells apart in a stable suspension is shown by the great difficulty ordinarily encountered in centrifuging live bacteria in a stable suspension into a mass from which the liquid can be decanted. On a basis of these deductions, it is possible to understand why agglutination of bacteria may take place when the cells exhibit the signs of possessing a charge, and also why agglutination might not take place when no repelling charge is present, as was reported by Northrop and De Kruif.³ The above deduction demonstrates that the surface energy (or surface tension, if you like) is the fundamental factor in the stability of suspensions that may either hold the cells apart or clump them together.

From the foregoing considerations we are led to the belief that the effect of the electric charge on suspensions of bacteria and on collloid particles in general in determining the stability of suspensions and degree of dispersion has been overestimated as a direct influence when a force due to electric charge repulsion is considered. The charge on such cells or particles must be of the nature of a Helmholtz double layer, and we believe that this double layer will affect the stability of the suspensions only so far as it will affect the surface energies. We interpret the findings of Northrop and De Kruif concerning a decrease in the electric charge on agglutination on this basis.

Some question might be raised as to the adsorption of substances at the cell-cell interface in a stable suspension. the condition necessary to bring about agglutination, in that no such interface exists in a stable suspension. It is to be borne in mind that adsorption at the two interfaces is only relative. A substance may be and probably is adsorbed in both when they exist; it is only necessary that the effect of reducing the specific energy of the interfaces be more in one than in the other to bring about a change in the equilibrium state. Let us consider a hypothetical case of agglutination by the mechanism developed above. The suspension is stable, no cells in contact, and the free energy is at a minimum. Now let us add to the system some substance capable of bringing about agglutination by adsorption. Let us assume that at first it is absorbed in the liquid surface in contact with the cell, the ectozone $S_{\rho t}$ reducing its specific energy e . The suspension is so far apparently more stable. If, however, this substance is capable of lowering the specific energy of the cell surface zone $S_{\rho_1 t_1}$, it will be adsorbed in the cell surface reducing e_2 . If the decrease in e_2 is greater than the decrease in e , the suspension is no longer in a state of equilibrium, for

if two cells come in contact with the formation of a cell-cell interface, the free energy of the system will become less. Consequently, as soon as two cells move to collide under the influence of brownian movement, they will not be held apart but will be drawn together with a decrease in free energy. Thus the free energy of the system will be decreased as rapidly as cells come in close proximity to one another.

SUMMARY

The difference in electrical potential between bacterial cells and liquid does not appear to be the fundamental force active in agglutination or dispersion.

The following formula is deduced giving the distribution of surface energies in bacterial suspensions as a function of the rate of change of free energy with degree of dispersion — $dE = dS [k_2(e_2 - e_1) - k_1(e - e_0)]$.

It is shown that the distribution of surface energies determines whether bacterial cells in a liquid will exist in a dispersed state or in an agglutinated state.

It is pointed out that maximum adsorption must occur at certain interfaces to bring about dispersion or agglutination.

It is possible to have equilibrium in any degree of agglutination or dispersion, provided the distribution of surface energies satisfies the equation of equilibrium deduced.

DIFFERENTIAL TESTS FOR COLON-AEROGENES GROUP IN RELATION TO SANITARY QUALITY OF WATER

STEWART A. KOSER

*From the Microbiological Laboratory of the Bureau of Chemistry, Washington, D. C., and the
Department of Bacteriology, University of Illinois, Urbana*

The introduction of simple methods for separating the colon-aerogenes group into two main sections has led to the question of the application of these procedures in the routine determination of the sanitary quality of water. When these methods were first brought forward it was apparently assumed by many that the use of the methyl red and Voges-Proskauer tests to divide this group of organisms into Bact. coli and the Bact. aerogenes sections, the so-called fecal and nonfecal types, would automatically solve the difficulties in the interpretation of the presence of the colon group in water supplies. While it is true that these means of differentiation constitute a great advance, nevertheless the question whether they are entitled to a place in routine water analysis is still a disputed subject.

To secure data on this point, Winslow and Cohen¹ made a study of the relative occurrence of the several types of the colon group in waters of different sanitary quality. They found the percentage of the methyl red positive type practically the same in polluted, unpolluted and stored raw waters. Thus, in a collection of 255 colon group organisms it was found that 76% of those from unpolluted waters, 77% from polluted waters and 85% from stored raw waters were methyl red positive and Voges-Proskauer negative. They state: "Our study of a limited number of cultures isolated from polluted, non-polluted and stored waters does not therefore seem to show any connection between the type of organism and corresponding source." Greenfield² has reported the results with 405 colon group cultures isolated from the waters of Kansas. The methyl red positive type constituted 76% of the cultures from raw waters, 70% of the cultures from treated waters and 65% of the cultures from ground waters. In a later publication, Greenfield and Skourup³ state that during rain or high water periods the greater proportion of the colon-aerogenes organisms from raw water belonged to the methyl red negative, Voges-Proskauer positive type. During dry weather this was reversed, and the greater proportion then belonged to the Bact. coli type. This was especially noticeable when the raw water was subject to sewage pollution. Wood⁴ has recorded the results of the methyl red and Voges-Proskauer tests when applied to the examination of

Received for publication, April 11, 1924.

¹ Jour. Infect. Dis., 1918, 23, p. 90.

² Ibid., 1916, 19, p. 647.

³ Jour. Indust. and Engin. Chem., 1917, 9, p. 675.

⁴ Jour. Hyg., 1919, 18, p. 46.

a number of water supplies in England. The methyl red negative type was found in 66 of 200 samples containing lactose fermenters. Detailed results of these 66 samples are given, showing a relation between the sanitary survey and the results of the methyl red differentiation. This work would be more convincing, however, if the findings with the other 134 samples were included, since one wonders whether the correlation between type of organism and source was evident in these samples. In conclusion, Wood states that the presence of organisms of the methyl red negative, Voges-Proskauer positive type should be regarded with less disfavor than the *Bact. coli* type, and he suggests that this differentiation be employed in all routine examinations of water and food products. Levine⁵ also believes this differentiation to be of value, for in his summary of the significance of lactose fermenters in water he states that it is obviously desirable, as it may assist in the detection of the probable source and nature of the contamination.

Rogers⁶ studied the ratio of *Bact. coli* to *Bact. aerogenes* in several small streams receiving sewage pollution. Just below sewer outlets the *Bact. coli* predominated, but farther down stream, as the distance from the source of pollution increased, the *aerogenes* types gained the ascendancy; that is, the ratio of the *coli* and *aerogenes* types correlated with the proximity of sewage pollution. It is noteworthy, however, that in several other water supplies Rogers found organisms of the *Bact. coli* type (methyl red positive) where the possibility of fecal pollution seemed slight. He states: "There is also the possibility that the digestive tract of animals is not the only source of the so-called fecal type of colon. At the present time there is little or no evidence that this is the case." We find a somewhat similar idea expressed by McCrady⁷ as a result of the examination of water supplies in Canada. He reports the frequent finding of methyl red positive Voges-Proskauer negative organisms in far greater numbers than could be accounted for by the sanitary survey, and he considered it probable that these organisms were not identical with true *Bact. coli* of fecal origin. He attempted to differentiate these forms from the typical fecal *Bact. coli*, but without success.

At the present time, it is generally agreed that the methyl red positive Voges-Proskauer negative type constitutes approximately 95% of the colon group organisms found in the feces of man and warm-blooded animals. On the other hand, it has been repeatedly shown that *Bact. aerogenes* and its allies predominate in soil and on grains. At first glance, the application to sanitary water analysis would seem to be simple, but it is here that the disagreement is most evident, and there is still a dispute as to the significance which may be placed on these types when encountered in water.

While studying the utilization of the commoner organic acid salts by members of the colon-aerogenes group, it was found⁸ that *Bact. coli* of fecal origin was incapable of utilizing sodium, potassium, or ammonium citrate, while *Bact. aerogenes* and its allies attacked the citrate.

⁵ Engin, Exper. Sta. Bull. 62, Ames, Iowa.

⁶ Jour. Bacteriol., 1918, 3, p. 313.

⁷ Bull. Sanitaire, 1916, 16, p. 103.

⁸ Koser, S. A.: Jour. Bacteriol., 1923, 8, p. 493.

In a simple synthetic medium containing the citrate as the only source of carbon, the typical fecal *Bact. coli* refused to develop, while the aerogenes section produced a visible turbidity, usually within 24 to 48 hours. So far as the typical fecal *Bact. coli* type and the aerogenes section are concerned, this differentiation correlates with the methyl red and Voges-Proskauer tests. In subsequent work⁹ this differentiation was applied to members of the colon-aerogenes group obtained from soils. Among these cultures were a number which were consistently methyl red positive and Voges-Proskauer negative, although they had been obtained from soils regarded as being free from pollution. When tested in the citrate medium, practically all of these methyl red positive soil cultures developed, as did *Bact. aerogenes* and its allies; that is, methyl red positive organisms from soil could be differentiated from the methyl red positive type from feces by the ability of the former to utilize citrate. As this differentiation was found to be quite constant, it is evident that the alimentary tract of warm-blooded animals may not be the sole source of the methyl red positive types encountered in nature outside of the body.

These methyl red positive soil forms which utilize citrate were encountered rather frequently, and it is reasonable to suppose that they may find their way into water supplies and there cause confusion in the usual interpretation of the methyl red and Voges-Proskauer tests. This would be especially true if a water supply were free from sewage contamination, since the original soil types would then not be obscured by other types indicative of pollution. There is also the possibility that in several of the former studies of water supplies these methyl red positive soil organisms may have been encountered in considerable numbers and may have contributed to the finding of approximately equal proportions of methyl red positive cultures in both polluted and unpolluted waters. If the foregoing assumptions are correct, then the test of citrate utilization to distinguish fecal *Bact. coli* from other members of the colon group may be of some value in clearing up the disputed points surrounding the significance of these types in water.

The aim of the present investigation has been to throw some light on the value of the citrate test when applied to water examination and also to compare these results with those of the other methods previously used for separating the members of the colon-aerogenes group.

The water supplies used in the present study fell into two groups. The first consisted of samples secured from sources which were shown

⁹ Ibid., 1924, 9, p. 59.

by sanitary survey to be apparently free from pollution, while the second was represented by obviously polluted water. Several small brooks and springs in Maryland and Virginia served as the source of the samples of high sanitary quality and the Potomac River at Washington, D. C., supplied the polluted samples.

In collecting samples which were judged free from pollution, every effort was made to make the sanitary survey as complete as possible. Most of these samples were taken from small brooks and springs in a hilly wooded country. In every case the surrounding territory and the drainage area were gone over carefully to insure absence of dwellings, camps or other possible sources of pollution. Small streams were followed up to their sources, and the surrounding territory was inspected before the samples were accepted as representing water of high sanitary quality.

For obtaining colon group organisms from the polluted waters, the synthetic plating medium of Ayers and Rupp¹⁰ was used. This permitted the plating of 1.0 and 0.1 c.c. quantities of water without preliminary enrichment in lactose broth. This method of isolation seemed preferable to the enrichment method, since one cannot be sure that the proportion of the various types of colon group organisms obtained from the lactose broth tubes is the same as that in the original sample of water. A few of the samples of polluted water were subjected to both lactose broth enrichment and to direct plating. The proportion of the several sections of the colon group obtained by these procedures was approximately the same in both cases. In the examination of the samples representing water of high sanitary quality, it was necessary to use lactose broth enrichment, since frequently colon group organisms could not be obtained from the small quantities of water used for direct plating. Quantities of water varying from 0.01 c.c. to 100 c.c. were used for this procedure. The smaller amounts were added directly to Durham fermentation tubes, while to the larger quantities sterile solutions of peptone, meat extract and lactose were added in sufficient quantities to bring the proportion of these ingredients to that usually used in lactose broth.¹¹ After incubation at 37 C. for 24 and 48 hours, all samples exhibiting fermentation were streaked on Endo plates, and from the plates showing development of colon-like colonies cultures were obtained for future study. When the colon-like colonies appearing on Endo plate were apparently all of one type, one culture only was

¹⁰ *Ibid.*, 1918, 3, p. 433.

¹¹ *Standard Methods of Water Analysis*, Am. Public Health Assn., 1923.

retained from that plate. Occasionally, however, two or perhaps more types of lactose-fermenting colonies were in evidence. In these cases, one representative of each type was kept; that is, if on the examination of a specimen of water, fermentation was noted in the 100 c.c. and 10 c.c. samples and the plate made from 100 c.c. sample showed apparently 2 types of colon group colonies, while that from the 10 c.c. sample exhibited only 1 type, then 3 cultures in all were retained from that particular specimen of water. Before further study all strains were subcultivated in lactose broth and replated on Endo agar. At the same time, agar slants of each culture, 2 to 4 days old, were examined by means of the Gram stain to detect the presence of any spore-formers. None were encountered in the present work.

It is realized that several objections may be raised to the foregoing procedures. In the first place, different methods were used for obtaining colon-like organisms from the two classes of water. However, as will be brought out later, this apparently does not obscure certain relations between each of the differential tests and the source of the water supplies. In the second place, the temperature of incubation was 37 C. It is possible that certain of the colon group cultures which do not develop readily or do not ferment lactose readily at this temperature were excluded by this procedure. Also, those strains which exhibit a delayed lactose fermentation were certainly excluded by an incubation period of only 48 hours. However, the present methods of water analysis call for incubation of the lactose broth at 37 C. for 48 hours, and since this custom is almost universal in water work it was followed here.

Four methods were employed to separate the principal sections of the colon-aerogenes group, namely, the methyl red test, Voges-Proskauer reaction, a synthetic uric acid medium and a synthetic citrate medium. For the methyl red and Voges-Proskauer tests, tubes of 0.5% dipotassium phosphate, Witte peptone and dextrose were incubated at 30 C. for 4 days before testing. The uric acid medium and the citrate medium were similar to those previously described.^{8, 9, 12} In these mediums the occurrence of growth was noted after 3 days' incubation at 30 C. All cultures obtained from the water samples were kept on plain nutrient agar for from 6 months to 1 year and tested several times during this period by each of the differential tests. The interval between isolation of the cultures from water and application of the differential tests for the first time varied from 1 to 5 weeks.

¹² Koser, S. A.: *Jour. Infect. Dis.*, 1918, 23, p. 377.

From the various water samples, 197 colon group cultures were obtained, 107 from polluted water and 90 from water accorded a high sanitary rating. The results of the differential tests applied to these cultures are shown in table 1. Here it is seen that the proportion of methyl red positive organisms found in the two classes of water is almost the same, for in the polluted samples this group constituted 80.4% of the total number of cultures and in the unpolluted waters 73.3%. The difference in the sanitary quality of these two classes of water, as shown by the sanitary survey, was great, and it is not brought out in the slight difference in the proportion between these two types.

The Voges-Proskauer test gives the same results. Again there is no marked difference between the two classes of water. If we are to consider all methyl red positive and Voges-Proskauer negative cultures as indicating intestinal origin, then we must conclude that about the

TABLE 1
RESULTS OF DIFFERENTIAL TESTS

	Polluted Water (Potomac River) 107 Cultures		High Sanitary Quality (Various Springs and Brooks) 90 Cultures	
Acid to methyl red.....	86	(80.4%)	66	(73.3%)
Voges-Proskauer negative.....	86	(80.4%)	70	(77.8%)
Uric acid negative.....	72	(67.3%)	60	(66.7%)
Citrate negative.....	69	(64.5%)	15	(16.7%)

same proportion of fecal cultures may be found in both polluted and unpolluted samples. It is true that in the case of the polluted samples the colon group organisms were usually detected in much smaller quantities of water than in the case of the unpolluted samples. Nevertheless, we would hardly expect to find a similar proportion of true *Bact. coli* of intestinal origin in these two diverse classes of water.

Also, in the case of the uric acid medium, there was no difference shown between the two classes of water. This is perhaps to be expected, for in a previous investigation⁹ it was found that the results given by the uric acid medium agreed more closely with the methyl red and Voges-Proskauer tests than with the citrate test.

When we come to differentiation on the basis of citrate utilization, there is a marked difference in the results. Of the cultures from the polluted samples, 64.5% failed to develop in the synthetic citrate medium and thus agreed with the *Bact. coli* of fecal origin, while only 16.7% of the cultures from unpolluted waters failed to utilize the citrate. If we accept the inability to utilize citrate as indicating fecal origin, then these

results are more in accord with the known quality of the water supplies than are those of the other differential tests.

This contrast can perhaps best be appreciated by considering a number of the individual water supplies. These are given in table 2. Since

TABLE 2
DIFFERENTIAL TESTS APPLIED TO REPRESENTATIVE CULTURES FROM VARIOUS CLASSES OF WATER

Source	Number	Quantity of Water From Which Obtained	Differential Tests			
			Methyl Red	Voges-Proskauer	Uric Acid	Citrate
Five different springs, all apparently unpolluted	1	10 c c.	+	0	0	+
	2	10 c c.	+	0	+	+
	3	0	+	+	+
	4	100 c c.	+	0	?	+
		10 c c.	0	+	+	+
		1 c c.	+	0	+ sl.	+
		1 c c.	+	0	0	+
	5	10 c c.	+	0	0	+
Several samples taken at different locations along a small brook; apparently unpolluted	1c-1	100 c c.	+	0	0	0
	1c-2	10 c c.	+	0	0	+
	2c-1	100 c c.	+	0	0	0
	2c-2	100 c c.	0	+	+	+
	2c-3	10 c c.	+	0	+	+
	3c-1	100 c c.	+	0	0	+
	3c-2	100 c c.	Neutral tints	0	+	+
	4c-1	100 c c.	+	0	0	+
	4c-2	1 c c.	+	+ or 0*	0	+
Potomac river at Washington, D.C., polluted	..	From various 1.0 and 0.1 c c. samples	+	0	0	0
			+	0	0	0
			+	0	0	0
			+	0	0	0
			0	+	+	+
			+	0	0	0
			0	+	+	+
			+	0	0	0
			+	0	0	0
			+	0	0	0
			+	0	0	0
			+	0	0	0
			+	0	0	0
			0	0	0	0
			0	+	+	+
Controls:	Bact. coli (Human, fecal)	1	+	0	0	0
		2	+	0	0	0
		10	+	0	0	0
		18	+	0	0	0
		25	+	0	0	0
	(Rabbit)	12	+	0	0	0
		16	+	0	0	0
	(Dog)	2	+	0	0	0
		3	+	0	0	0
	Bact. aerogenes (soil and water)	Va. 3	0	+	+	+
		Md. 2	0	+	+	+
		P	0	+	+	+
		RC-5	0	+	+	+

* Variable results shown on repeated tests; +sl., very light turbidity evident in uric acid medium.

the results are given here in detail, the number of cultures shown in table 2 is limited. Only a few colon group organisms were obtained from springs which were apparently unpolluted. All of the cultures from springs are included in the table, and their behavior in the citrate medium is compared with that of representative cultures isolated from other classes of water. All of the 8 cultures from spring water utilized citrate, although 6 of them were methyl red positive. Thus, on the basis of citrate utilization, all of these methyl red positive cultures can be differentiated from the typical fecal *Bact. coli* shown in the controls at the bottom of the table. In the second series, 7 of the 9 cultures obtained from a small brook were methyl red positive, while only 2 agreed with the fecal *Bact. coli* in their deportment in citrate. Again the majority of the methyl red positive cultures are distinct from the intestinal type, and the results of the citrate test are more in harmony with the known sanitary quality of the water. The cultures from the Potomac river water are in contrast to those of the foregoing 2 groups in that most of the methyl red positive organisms fail to utilize citrate and thus resemble intestinal *Bact. coli*.

It is noteworthy that in each class of water there is found about the same proportion of methyl red positive cultures. On the other hand, the citrate test gives results which are more in keeping with the sanitary survey of the respective water supplies, and the proportion of citrate negative cultures increases as we go from unpolluted springs on the one hand to the fecal *Bact. coli* controls on the other. It should also be noted that the two citrate negative cultures from the small brook were recovered from large amounts of water, 100 c.c. in each case, whereas smaller quantities of water yielded organisms distinct from the fecal type. It is uncertain whether these two cultures were in reality soil organisms which, contrary to the general rule, failed to utilize citrate or whether they represent actual fecal cultures. It should be borne in mind that in the case of small brooks the possibility of pollution from wild animals and birds cannot be excluded and may account for an occasional organism of the intestinal type.

The results of the methyl red and Voges-Proskauer tests are especially interesting in view of the previous investigation by Winslow and Cohen,¹ who found that the methyl red positive type constituted 76% of the colon group organisms obtained from unpolluted waters and 77% from polluted waters in the vicinity of New Haven. In the present investigation, based on water in the vicinity of Washington, D. C., we

find 73.3% from unpolluted and 80.4% from polluted waters to be methyl red positive. This similarity of results obtained in different sections of the United States is quite striking, and our conclusion in regard to these tests is in agreement with that formerly expressed by Winslow and Cohen, namely, the methyl red and Voges-Proskauer tests have failed to bring out any noteworthy correlation between type of organism and corresponding source. The same may also be said of the uric acid test.

The promising results shown by the citrate medium indicate that this method of differentiation is deserving of further study with regard to its usefulness and application in the sanitary examination of water supplies. The final acceptance of any such test must, of course, await general confirmation at the hands of different workers.

SUMMARY

Various tests to differentiate the members of the colon-aerogenes group were applied to two series of cultures, one obtained from polluted water and the other from water of high sanitary quality as shown by sanitary survey. The tests were the methyl red test, Voges-Proskauer reaction, the ability to develop in a synthetic uric acid medium and the ability to utilize citrate.

The proportion of methyl red positive cultures was found to be almost the same in each class of water. These organisms constituted 80.4% of the colon group cultures obtained from polluted water and 73.3% of those from unpolluted water. In a similar manner, the results of the Voges-Proskauer reaction and of the uric acid test failed to correlate with the sanitary survey of the water supplies.

The test of citrate utilization, on the other hand, showed some degree of correlation with the sanitary survey of the water supplies. Citrate negative cultures (similar to fecal *Bact. coli*) constituted 64.5% of the colon group organisms from polluted water and 16.7% of those from waters of high sanitary quality. This correlation was shown also by a consideration of several of the individual water supplies.

SPECIFIC AND NONSPECIFIC PROPERTIES OF WASSERMANN ANTIGENS

EMIL WEISS AND LLOYD ARNOLD

*From Department Bacteriology, Pathology and Preventive Medicine, Loyola University
School of Medicine, Chicago*

Kolmer¹ has recently compared various methods and procedures used in the Wassermann reaction and has recommended a standard technic. In view of the confusion that has existed in this field, Kolmer's contribution has been of great value in helping to establish a systematic and uniform procedure. His methods for the technic of the Wassermann test are excellent, but the method of selection of the antigenic dose that he recommends is a variable and uncertain amount. It requires a separate titration almost each time the antigen is needed, and we have found this to be a changeable quantity, depending mostly on the temperature at which the stock antigen was preserved and on the method of dilution. Great variations occur when the exact technic, recommended by Kolmer, is followed.

The antigen or antigens used in the Wassermann reaction have been studied more than any other component. The instability of the first aqueous extracts used for antigens led to the use of the more stable alcoholic, acetone, ether, etc., extracts.

Kiss² was the first to point out clearly the effects that these solvents exerted on the hemolysis of the red blood cells. Kaup³ studied in detail the specific and nonspecific properties of various antigens. He found by gradually decreasing the amount of extract, the following zonal reactions: (1) precipitation, (2) hemolysis, (3) inhibition, (4) specific and (5) inefficient zones. After evaporating one half of the alcohol from his extracts and making up to volume with normal salt solution, he found with the same amount of this extract an absence of precipitation, with the other reactions present in the order named above. The antigen dose that did cause inhibition would, with a diminution of the alcoholic content of the extract, cause a specific reaction with positive serum.

Received for publication, April 15, 1924.

¹ Am. Jour. Syphilis, 1922, 6, p. 651.

² Ztschr. f. Immunitätsf., 1910, 4, p. 703.

³ Kritik der Methodik der Wassermannschen Reaktion und neue Vorschläge für quantitative Messung der Komplementbindung, 1917.

Kaup concluded that the antigenic properties of an alcoholic extract were due to the combined influence of the alcohol and the extracted substances.

We have studied the specific and nonspecific properties of antigens by using the following extracts: alcoholic extracts of syphilitic liver, normal human liver, human heart, beef heart, cholesterinized beef heart and guinea-pig heart; acetone extracts of syphilitic liver and beef heart; acetone insoluble lipoids and Lesser's ether extract. We have used various methods of preparation, such as varying the ratio between the amount of tissue—both wet and dry—and the solvent used; also the time, temperature, etc., of the extraction, and many other modified methods of procedure. We wish to show in this paper that the titration of the antigens and the selection of the proper doses is the main factor in its usefulness and not so much the particular method of preparation; if properly titrated, many weak antigens are as useful as stronger ones.

Careful titration of antigenic extracts shows that each and every one has certain characteristic zonal reactions. There are roughly 5 large zones: the precipitation, hemolytic, inhibitory or anticomplementary, specific or antigenic, and the inefficient zone. By making use of this colloidal chemical zonal phenomenon, we think valuable information can be obtained as to the usefulness of any antigen for the Wassermann reaction work.

Roughly speaking, the precipitation and the hemolytic zones are nonspecific and are due more to the solvent than to the dissolved substances. The inhibitory or anticomplementary zone is effected more by the dissolved substances in the extract than by the solvent itself. The specific or antigenic zone is the one that is most important to the serologist. This zone is influenced to a great extent by the dissolved substances and is little effected by the solvent itself. The lowest zone is produced as a result of the dilutions, when these are too small to be effective, the inefficient zone begins.

PRECIPITATION ZONE

This precipitation is produced with the largest doses of the extract. It is not influenced to an appreciable extent by the addition of serum, complement of amboceptor. Tables 1 and 3 show the extension of this zone under various conditions. It is due primarily to the presence of the solvent used in the extraction.

HEMOLYTIC ZONE

This hemolysis is produced for the most part by the solvents used for the extraction. The dissolved substances tend to shorten this zone, the more concentrated the extract the narrower is the zone (tables 1 and 2).

INHIBITION OR ANTICOMPLEMENTARY ZONE

Inhibition is influenced more by the extracted substances than the solvents. This zone is changed more than the precipitation and hemolytic zones by the addition of serum, amboceptor or complement; all of

TABLE 1

THE INFLUENCE OF LIPOIDS AND SOLVENTS ON THE REACTION OF AN EXTRACT

Total volume 2.5 c.c., 0.5 c.c. 10% complement, 4 units amboceptor and 0.5 c.c. 5% suspension of washed sheep blood cells. Incubation each time for a period of 1 hour at 37 C.

Alcoholic Beef Heart Extract	Doses of Antigen in C.c.											
	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.15	0.1	0.05	0.01	0.005
With 0.1 c.c. normal serum.....	P	P	trL	L	I	I	I	i.I	L	L	L	L
With 0.1 c.c. syphilitic serum.....	P	P	trL	L	I	I	I	I	I	I	i.I	L
Alcoholic content reduced to one half* with 0.1 c.c. normal serum..	P	trL	I	I	i.I	L	L	L	L	L	L	L
Alcoholic content reduced to one half* with 0.1 c.c. syphilitic serum	P	trL	I	I	I	I	I	I	I	i.I	i.I	L
Alcohol completely removed* with 0.1 c.c. normal serum.....	I	I	I	i.I	L	L	L	L	L	L	L	L
Alcohol completely removed* with 0.1 c.c. syphilitic serum.....	I	I	I	I	I	I	I	I	I	i.I	L	L
Cholesterolized (0.2%) with 0.1 c.c. normal serum.....	P	P	P	L	trL	I	I	I	L	L	L	L
Cholesterolized (0.2%) with 0.1 c.c. syphilitic serum.....	P	P	P	L	trL	I	I	I	I	I	I	i.I
Lipoids 5 times increased with 0.1 c.c. normal serum.....	P	P	P	P	P	P	trL	I	I	i.I	L	L
Lipoids 5 times increased with 0.1 c.c. syphilitic serum.....	P	P	P	P	P	P	trL	I	I	I	I	I

P indicates precipitation; L, hemolysis; I, inhibition; tr, trace; i, incomplete.

* Made up to original volume with normal salt solution.

these cause a widening or increased extension of the zone in the titration tubes. (table 2). It is of practical importance to divide this zone into two parts. Complete inhibition of both normal and syphilitic serums forms the upper subzone, incomplete inhibition with normal and complete inhibition with syphilitic serums forms the lower subzone. The significance of these subzones will be mentioned later.

SPECIFIC OR ANTIGENIC ZONE

Certain doses, smaller than those causing inhibition, produce hemolysis with a normal serum, in the presence of complement and ambo-

ceptor, while inhibition results with the use of syphilitic serum. The amount of the extract used causing this reaction lies within the specific antigenic zone. Not all doses within the specific zone show with a syphilitic serum the same degree of inhibition. Some smaller doses give only incomplete inhibition. We can divide this zone into two parts, an upper and a lower subzone. The upper subzone gives hemolysis with normal serum and complete inhibition with syphilitic serum, the incomplete inhibition is usually found in serum of latent syphilitic patients or after treatment, etc. The lower subzone gives hemolysis with normal serum; incomplete inhibition with syphilitic serum, that showed complete inhibition in the upper subzone; hemolysis or only slight inhibition with positive serum, that showed incomplete inhibition in the upper subzone. It will be seen from table 2 that the specific zone is widest with the lipid-normal salt suspension. The extracts show a narrower zone, and the solvent alone shows only an abbreviated upper subzone, and this disappears on further incubation (table 3). Only the amounts use that come within the upper subzone of the specific zone are of practical value in the Wassermann reaction.

INEFFICIENT ZONE

Doses of extracts or their component parts smaller than those causing the specific zonal reaction show hemolysis with both normal and syphilitic serums. Hemolysis in this instance is caused through the combined influence of complement and amboceptor, while the hemolysis that occurred in the hemolytic zone was mostly due to the action of the solvents.

FACTORS INFLUENCING THE LOCALIZATION OF ZONES

These can be briefly summed up as follows:

Solvents.—These produce a more distinct reaction in the precipitation and hemolytic zones. The zonal reactions are all produced in a shorter period of time with solvents than with antigens free of solvents.

Extracted Substances.—These substances produce the precipitation and hemolytic only after longer incubation; the inhibition and specific zones are much broader and better defined than in the case of the extracts or solvents. An increase of these extracted substances causes a shifting of all zones toward the lower doses and a narrowing of the hemolytic zone.

TABLE 2

THE INFLUENCE OF SERUM ON THE REACTION OF THE EXTRACT* AND THEIR COMPONENTS

Total volume 2.5 c c.; 0.5 c c. 5% suspension of washed sheep blood cells; incubation each time for a period of 1 hour at 37 C. (incubator).

	Doses of Antigen in C c.												
	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.16	0.12	0.1	0.05	0.01	0.005
Extract													
Alone.....	P	P	P	trL	trL	I	I	I	I	I	I	I	I
With 0.1 c c. serum.....	P	P	trL	trL	I	I	I	I	I	I	I	I	I
With 0.1 c c. serum.....	P	P	P	trL	I	I	I	I	I	I	I	I	I
With 0.1 c c. normal serum, 0.5 c c. 10% complement and 4 units amboceptor..	P	P	trL	trL	I	I	I	i.I	i.I	L	L	L	L
With 0.1 c c. syphilitic serum, 0.5 c c. 10% com- plement and 4 units am- boceptor.....	P	P	trL	trL	I	I	I	I	I	I	I	I	i.I
With 0.5 c c. normal serum, 0.5 c c. 10% complement and 4 units amboceptor..	P	P	P	trL	I	I	I	I	i.I	i.I	L	L	L
With 0.5 c c. syphilitic serum, 0.5 c c. 10% com- plement and 4 units am- boceptor.....	P	P	P	trL	I	I	I	I	I	I	I	I	I
Lipoids													
Alone.....	I	I	I	I	I	I	I	I	I	I	I	I	I
With 0.1 c c. serum.....	I	I	I	I	I	I	I	I	I	I	I	I	I
With 1 c c. serum.....	I	I	I	I	I	I	I	I	I	I	I	I	I
With 0.1 c c. normal serum, 0.5 c c. 10% complement and 4 units amboceptor..	I	I	I	I	i.I	i.I	L	L	L	L	L	L	L
With 0.1 c c. syphilitic serum, 0.5 c c. 10% com- plement and 4 units am- boceptor.....	I	I	I	I	I	I	I	I	I	I	I	I	i.I
With 0.5 c c. normal serum, 0.5 c c. 10% complement and 4 units amboceptor..	I	I	I	I	I	i.I	i.I	L	L	L	L	L	L
With 0.5 c c. syphilitic serum, 0.5 c c. 10% com- plement and 4 units am- boceptor.....	I	I	I	I	I	I	I	I	I	I	I	I	I
95% Alcohol													
Alone.....	P	P	L	L	L	I	I	I	I	I	I	I	I
With 0.1 c c. serum.....	P	trL	L	L	I	I	I	I	I	I	I	I	I
With 1 c c. serum.....	P	P	L	L	I	I	I	I	I	I	I	I	I
With 0.1 c c. normal serum, 0.5 c c. 10% complement and 4 units amboceptor..	P	trL	L	L	I	I	i.I	L	L	L	L	L	L
With 0.1 c c. syphilitic serum, 0.5 c c. 10% com- plement and 4 units am- boceptor.....	P	trL	L	L	I	I	I	i.I	L	L	L	L	L
With 0.5 c c. normal serum, 0.5 c c. 10% complement and 4 units amboceptor..	P	P	L	L	I	I	I	i.I	L	L	L	L	L
With 0.5 c c. syphilitic serum, 0.5 c c. 10% com- plement and 4 units am- boceptor.....	P	P	L	L	I	I	I	I	i.I	L	L	L	L

* Alcoholic extract human heart.

Extracts.—These usual antigenic extracts act like the solvents in the precipitation and hemolytic zones and like the solvent free saline suspensions in the inhibition and specific zones. Weak extracts react in general similarly to the extraction mediums, strong extracts similarly to the solvent-free saline antigens. Most extracts giving inhibition and hemolysis in relatively small doses are usually strong extracts, and when properly titrated are very useful antigens.

Serum.—The addition of small amounts of positive or negative serum (0.1 c c.) to decreasing doses of the extracts of their components causes a shifting of the precipitation, hemolytic and the upper part of the inhibition zones toward the larger doses. Larger amounts of positive or negative serums (0.5 c c., 1 c c.) exert the same influence but to a much lesser degree.

Complement.—The amount of complement usually employed in the Wassermann test (0.5 c c. of a 10%) exerts a similar influence as does serum. Larger amounts of undiluted complement (0.5, 1 c c.) act the same as the same amount of serum.

Ambocceptor.—The amount of ambocceptor used in the Wassermann test (2-5 units) does not exert any influence on the precipitation, hemolytic and upper part of the inhibition zones. The ambocceptor shows, in the presence of complement, in the upper inhibition subzone complete inhibition and in the lower inhibition subzone incomplete inhibition, in lower dilutions of antigen, only hemolysis.

Blood Cells.—Almost the same results can be obtained with 2.5 to 20% suspensions of red blood cells, in the precipitation and hemolytic zones, while in the inhibition, specific and inefficient zones the more concentrated suspensions cause an extension of these zones toward the smaller doses.

Temperature and Time of Incubation.—The duration of the incubation has an important bearing on the results. The longer the incubation or the higher the temperature the more pronounced are the precipitation and hemolytic zones, at the same time the specific and inefficient zones progress toward the larger antigenic doses. This causes a narrowing of the inhibition zone and sometimes leads to its total disappearance (table 3).

DISCUSSION

We do not wish to go into a theoretical discussion of this zonal phenomenon at this time. We wish to emphasize the practical importance of the knowledge of the zonal reactions of an antigen. The

precipitation, hemolytic, inhibition and inefficient zones are nonspecific for syphilis, but a knowledge of these different zonal reactions is necessary for an accurate understanding of the antigen. If, in the titration of an antigen, with progressively decreasing dose, with normal and syphilitic serums, we find the specific or antigenic zone narrow and ill-defined, we are dealing with a weak extract. Such an extract contains little extractable substances of antigenic value; the zonal reactions are similar to the same reactions produced by the solvent alone in the

TABLE 3

THE INFLUENCE OF TEMPERATURE AND DURATION OF INCUBATION ON THE REACTION OF THE EXTRACT* AND THEIR COMPONENTS

Total volume 2.5 c.c.; 0.5 c.c. 5% suspension of washed sheep blood cells.

	Doses of Antigen in C.e.									
	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.16	0.12	0.1-0.05
Extract										
Icebox temperature 1 hour.....	P	trL	trL	trL	I	I	I	I	I	I
Icebox temperature 12 hours.....	P	P	P	trL	trL	I	I	I	I	I
Room temperature 1 hour.....	P	P	trL	L	iI	I	I	I	I	I
Room temperature 12 hours.....	P	P	P	P	trL	trL	trL	I	I	I
Incubator at 37 C. 1 hour.....	P	P	P	trL	L	iI	I	I	I	I
Incubator at 37 C. 12 hours.....	P	P	P	P	P	P	trL	trL	I	I
Lipoids										
Icebox temperature 1 hour.....	I	I	I	I	I	I	I	I	I	I
Icebox temperature 12 hours.....	I	I	I	I	I	I	I	I	I	I
Room temperature 1 hour.....	I	I	I	I	I	I	I	I	I	I
Room temperature 12 hours.....	P	trL	I	I	I	I	I	I	I	I
Incubator at 37 C. 1 hour.....	I	I	I	I	I	I	I	I	I	I
Incubator at 37 C. 12 hours.....	P	P	P	P	trL	I	I	I	I	I
Methylalcohol										
Icebox temperature 1 hour.....	L	L	L	iI	I	I	I	I	I	I
Icebox temperature 12 hours.....	P	P	L	L	L	I	I	I	I	I
Room temperature 1 hour.....	L	L	L	L	iI	I	I	I	I	I
Room temperature 12 hours.....	P	P	P	P	L	L	I	I	I	I
Incubator at 37 C. 1 hour.....	P	P	L	L	L	I	I	I	I	I
Incubator at 37 C. 12 hours.....	P	P	P	P	P	P	I	iI	I	I

* Acetone insoluble lipoids.

same or a little larger concentrations. If such an extract is carefully evaporated at a relatively low temperature (37 to 40 C.), and the resulting residue made up to one-half or three-fourths of the original volume with normal salt solution, the antigenic zone will be found to be broad enough to be useful in the Wassermann reaction.

In all alcoholic and acetone extracts, we have found that the evaporation of the solvent and the suspension of the residue in normal salt solution gave us a more useful antigen than the original extract.

There are three methods at present used in determining the antigenic dose by serologists.

(1) The anticomplementary unit is determined and the specific dose of antigen is a certain portion of this amount (Wassermann).

(2) The antigenic unit is determined and a certain multiple of this is taken as the antigenic dose (Kolmer).

(3) Complement is adapted to a fixed amount of antigen (Boas). The first two methods are the ones in general use. The antigenic dose in the first method may closely approach or even go into the inefficient zone. The second method may approach or extend into the anticomplementary zone. It is purely accidental if by either method the antigenic dose happens to be midway between the two extremes of the antigenic zone.

The extract should be preserved at the same temperature as that used in the extraction process. If the temperature is lower, a precipitate usually falls out that must either be removed by filtration or dissolved by heating; in either instance the antigenic titer is changed. We have found that the icebox affords the most constant temperature for the preparation and preservation of antigenic extracts. If a given amount of this extract is evaporated at 37 C. and the residue carefully suspended in normal salt solution, we have found this to be the most useful antigen for the Wassermann reaction. A uniform suspension is facilitated by adding warm saline (40 C.). After the titration of this residue—salt solution antigens—we take as the dose that amount midway between the two extreme ends of the upper antigenic or specific subzone. This is not an arbitrary amount, but is a fixed dose, and we know the margin of safety we have between this dose and that causing inhibition or anticomplementary action in larger doses and hemolysis in lower doses. The wider this specific or antigenic zone, the safer and the more useful is the particular antigen. This zone is widest when the solvent is removed and the residue suspended in salt solution.

This method does not depend on the addition of an alcoholic or acetone extract to salt solution by a drop method, the resulting suspension depending on temperature, the size of the drops, rapidity of addition of the drops, etc., and other unknown factors. Weak extracts do not need to be discarded. After evaporation the volume of salt solution added can be varied until the antigenic zone is wide enough to be of practical value. Once a proper titration is made, the same procedure yields constant results, and consequently one titration is sufficient; the antigen titer does not need to be made each time.

SUMMARY

Certain zonal reactions take place with the use of varying amounts of antigen in the Wassermann reaction. These are from the larger toward smaller doses, precipitation, hemolytic, inhibitory, antigenic and inefficient zones. Only the antigenic zone is specific with syphilitic serum. The main factor causing these nonspecific zones is the solvent used in the extraction. Antigens freed of the solvent by evaporation at low temperature and made up to the required volume with saline have proved most satisfactory. This residue—saline antigen—gives a wider specific zone. It is not as sensitive to temperature changes. It is stable and constant in its reactions. The antigenic dose used for the final Wassermann test should be the amount of this residue—saline antigen—that is half way between the largest and smallest dose causing hemolysis with normal serum and complete inhibition with syphilitic serum. This is a fixed titrable amount, and nonspecific reactions are excluded.

THE MYOCARDIAL LESIONS OF DIPHTHERIA

ALDRED SCOTT WARTHIN

From the Pathological Laboratory of the University of Michigan, Ann Arbor, Mich.

For nearly seventy-five years, the attention of clinicians has been directed to the involvement of the heart in diphtheria, and their interest in the cardiac manifestations of this disease has steadily increased with the years. Particularly during the last 25 years has the clinical study of cardiac symptomatology in diphtheria taken on fresh momentum as the result of the application of the newer methods of cardiologic investigation. Today all internists are agreed concerning the serious import of symptoms of cardiac disturbance developing during or after the course of this infection. The frequency of such cardiac involvement is shown by numerous clinical statistics. Osler¹ stated that irregularity of the heart occurs in 60% of the nonfatal cases, while murmurs at the apex or base are found in 94%. Other clinicians hold that some degree of cardiac injury is shown in every case of the disease. As death from obstruction of the respiratory tract has diminished in frequency as the result of improved methods of treatment, death from heart failure has come to be the most common form of death in diphtheria.

The history of diphtheria as a clinical entity covers about 100 years. Bretonneau,² in 1821, established the first definite clinical criteria for this infection under the name of angina diphtheritis, basing his conception on the essential character of the exudation. He at first held that it was a purely local infectious process, but later recognized a blood-poisoning as one of its essential characteristics. It seems strange that he failed to discover the cardiac involvement; for he insisted that the cause of death was always asphyxia or suffocation from the obstruction of the respiratory passage through the formation of a pseudo-membrane in the larynx or trachea. Various clinicians following him, among them his scholar, Trousseau,³ observed cases in which collapse occurred without any laryngeal or tracheal membrane, and that it also occurred after tracheotomy. Through the fourth and fifth decades of the nineteenth century, the suspicion that the heart was directly con-

Received for publication, April 18, 1924.

¹ Principles and Practice of Medicine, 1920, p. 780.

² Mem. d. l'Acad. d. Méd., 1821.

³ Med. Clin. d. Hôtel Dieu, 1866, p. 434.

cerned in the production of such collapse came to be more and more entertained by the physicians of the period. Greater stress was laid also on the general affection, and on such symptoms as paralysis of the muscles of the throat and other parts of the body. It was but natural that the conception of cardiac paralysis should arise as the first explanation of the cases of collapse in diphtheria. Werner,⁴ in 1842, seems to have been the first observer to connect pathologic conditions of the heart with sudden death in diphtheria. Through the fifties numerous clinical observations on the rôle played by the heart in death from diphtheria were made, and by 1860 at least two working hypotheses had been advanced: cardiac paralysis, and the formation of coagula within the heart cavities.

It is of great interest to trace the development of the theories of cardiac pathology in diphtheria from 1860 to the present time. It presents an evolution closely paralleling that of the general theory and science of medicine during this period. The phases of this evolution may be roughly classed as follows: (1) cardiac paralysis, (2) cardiac thrombosis, (3) infectious endocarditis, (4) endarteritis of cardiac vessels, (5) infectious parenchymatous myocarditis, (6) infectious interstitial myocarditis, (7) injury to cardiac nerves and ganglions, vagus, abdominal sympathetic and vasomotor system, resulting in cardiac paralysis, (8) toxic myocarditis, (9) special affinity of diphtheria toxin for impulse-conducting system of heart (bundle of His).

Following the historical development of these different theories, we find that Werner⁴ was the first to connect the occurrence of fibrinous concretions within the heart with sudden death in diphtheria. Ten years later, Winkler⁵ made similar observations. The clinical importance of such coagula in the heart chambers in diphtheria was especially urged by the English school. Richardson⁶ thought that the clots found in the heart in fatal cases of diphtheria were the result of an excess of fibrin in the blood, leading to a deposit in the heart analogous to the diphtheric deposits in the membranes in the pharynx and larynx; and that death through syncope resulted from the obstruction of the chambers of the right side of the heart by such coagula. He differentiated the symptoms of cardiac obstruction from those of respiratory obstruction. In the former the body is cold, the skin pale, almost like marble, especially over the extremities; the lips are slightly blue; the jugular veins are distended; the pulse, very irregular, quick, feeble; heart sounds, muffled with a bruit in some cases; there is intense anxiety and constant movement, but no real convulsions. Beau⁷ saw similar cases of cardiac concretions and ascribed the sudden death to cardiac paralysis.

⁴ Cited by Leyden.³⁰

⁵ Die Blutklumpen in der heutigen Bräune, 1852.

⁶ Med. Times and Gaz., 1856, p. 231.

⁷ Gaz. d. hôp., 1858, 31, p. 165.

Barry,⁸ Smith,⁹ Bristowe¹⁰ and Thompson¹¹ made similar observations of the occurrence of cardiac syncope in diphtheria associated with the presence of fibrinous concretions in the heart cavities. Bristowe noted also their association with extravasations and fatty degeneration in the heart muscle. Thompson stated that his observations led him to believe that such clots were the most frequent cause of death in diphtheria in the second or third week of convalescence, and that their formation led to sudden faintness, pallor of the face and lips, vomiting, dyspnea, slow and feeble pulse, pain in the cardiac region, epileptiform convulsions and death in syncope. Meigs, in America¹² also saw cases leading him to believe that death in diphtheria was dependent on the formation of fibrinous concretions in the heart. He ascribed the production of membranes on the mucous surfaces and of concretions in the heart cavities to some especial alteration in the constitution of the tissues and the body fluids. In the same year, Hillier¹³ reported 3 cases in which death took place from heart failure 3 weeks after diphtheria, with evident degeneration of the heart muscle. Gerlier¹⁴ supported the view of cardiac thrombosis as a cause of death in diphtheria; and in the early seventies, a number of French writers upheld this view in a group of theses and other writings on this subject. Robinson¹⁵ considered cardiac thrombi to be of common occurrence in diphtheria and an important cause of death. He explained their formation as being due to a paralysis of the vagus and an increased tendency of the blood to coagulate (inopexia). He noted, however, that the heart muscle was often pale and easily crushed.

Bouchut¹⁶ noted waxy degeneration of the heart muscle and a proliferative endocarditis in association with fibrinous deposits on the valvular and parietal endocardium in two cases of diphtheria. He compared the muscle degeneration with that described by Zenker,¹⁷ Waldeyer¹⁸ and Hayem¹⁹ as occurring in typhoid fever; and concluded that the cardiac thrombosis seen in diphtheria was the result of an infectious endocarditis and myocarditis. In 1873, Labadie-Lagrave²⁰ published his thesis from Bouchut's clinic, based apparently on the same observations with a more detailed description of the endocardial changes. He described the auriculoventricular valves as injected and covered with small miliary deposits of fibrin. He concluded that thrombosis in the hearts of persons with fatal cases of diphtheria was the result of the endocarditis.

The view of an infectious vegetative thrombosis as a cause of death in diphtheria was taken up by many French and English writers, and this idea persists even in the literature of the present day. It met with little favor in Germany, where the cardiac coagula found in the hearts of persons with diphtheria had been pretty generally recognized as agonal clots, and of no significance beyond that of progressive cardiac insufficiency. Eberth's paper,²¹ which became widely quoted in association with the discussion on endocarditis in diphtheria, apparently had nothing to do with diphtheria, and related only to severe ulcerative endo-

⁸ Brit. Med. Jour., 1853, p. 623.

⁹ Med. Times and Gaz., 1859, 2, p. 617.

¹⁰ Med. Times, 1859, pp. 180 and 227.

¹¹ Med. Times and Gaz., 1860, 1, p. 23.

¹² Am. Jour. Med. Sc., 1864, 47, p. 148.

¹³ Med. Times, 1864, p. 204.

¹⁴ Thesis, Paris, 1866.

¹⁵ De la thrombose cardiaque dans le diphtherie, 1872.

¹⁶ Gaz. d. hôp., 1872, p. 937.

¹⁷ Ueber die Veränderungen d. milkkürlichen Muskeln in Typhus abdominalis, 1864.

¹⁸ Arch. f. path. Anat., 1865, 34, p. 473.

¹⁹ Mem. Soc. Biol., 1866; Arch. d. physiol. norm et path., 1870, 3, p. 69.

²⁰ Des comp. cond. du croup et de la diphtherie, 1873.

²¹ Arch. f. path. Anat., 1873, 57, p. 228.

carditis with "diphtheric" necrosis of the valvular surface. In France also the theory of an infective endocarditis in diphtheria met with opposition. Dufresne²² opposed the views of Robinson, Bouchut and Labadie-Lagrave in a critical thesis based on the examination of the heart in diphtheria. In half of the cases he examined he found coagula in the heart cavities, but never any signs of endocarditis. He regarded the coagula as either agonal or postmortal. In some cases, he noted slight and localized fatty degeneration of the heart muscle, but considered it doubtful whether it had any influence on the cardiac function. He rejected Labadie-Lagrave's cases and considered the valvular changes due to capillary extravasations due to asphyxia. While agonal cardiac coagula might lead to death, he declared that sudden death in diphtheria is as a rule due to tracheal obstruction. Parrot²³ found changes similar to those described by Labadie-Lagrave in the heart valves of young children dying from conditions other than diphtheria, and regarded them as having no especial relationship to diphtheria. Beau-Verdeney²⁴ also minimized the importance of endocarditis in diphtheria.

The pathologic and clinical knowledge of the heart in diphtheria was summed up by Oertel,²⁵ who regarded diphtheria as a primary local infection with involvement of the whole body from the primary focus; but gave little attention to the clinical manifestations of the cardiac complications. He notes the occurrence of hemorrhages in the myocardium and beneath pericardium and endocardium, and states that in death by suffocation the heart muscle may appear entirely unaltered in color and texture as well as in its histologic elements. In patients succumbing to general poisoning and septicemia, the muscle of the heart is more friable and shows extravasations and local collections of nuclei and cells between the fibers. In cases in which death is produced suddenly by paralysis of the heart, the myocardium appears pale, soft and friable, and broken by extravasations, and on microscopic examination, the majority of the fibers are found to be in an advanced stage of fatty degeneration.

During the latter part of the seventh and through the eighth decade, the majority of researches concerning diphtheria had for their object the determination of the bacterial causal agent of the infection, which was finally accomplished by Klebs (1883), Loeffler (1884), and Darier (1885). The growing conception of the bacterial origin of diphtheria had, as we have seen, been responsible, particularly in France and England, for the theory of an infectious diphtheric endocarditis. This conception of the cardiac involvement in the disease had met with but little sympathy in Germany, where the heart clots had been early recognized as agonal in character, the result of the cardiac weakness and not its cause. The pathologic anatomy and histology of the diphtheria heart now began to receive especial attention. The changes in the voluntary muscles in various infections (typhoid, variola, etc.) as described by Virchow, Zenker, Waldeyer, Hayem, Popoff, Fraenkel, Leyden, Oertel, Fritz and others, made it probable that such changes would be found also in the muscles of patients with diphtheria. As already noted, anatomic changes in the heart in fatal cases had been mentioned more or less casually by Bristowe, Hillier, Hoffmann and Hayem, Bouchut, Labadie-Lagrave and others in the form of parenchymatous degeneration (fatty, granular and waxy) or interstitial changes (increase of cells and nuclei); but no uniformity of interpretation had been reached.

There was now to be developed the theory of an infectious myocarditis based on the observations of Mosler, Rosenbach, Birch-Hirschfeld, Guttman, Leyden

²² Contributions a l'étude du Croup, 1873.

²³ Thesis, 1874; cited by Leyden.³⁰

²⁴ Etude critique de l'endocardite dans la diphtherie, 1874.

²⁵ Deutsch. Arch. f. klin. Med., 1871, 8, p. 345; Van Ziemssen's Handbook, 1872-3, 2.

and Huguenin, extending over the next 15 years (1873-1888). Mosler²⁶ was the first German writer who noted myocardial changes in cases of fatal collapse occurring in diphtheria in young children. In one case he found the heart dilated, with an aneurysmal dilatation of the left ventricle at the apex, the myocardium of a dirty pale color, fairly firm, but showing microscopically a marked fatty degeneration. In a second case with symptoms of collapse as described by Trousseau, the heart showed similar dilatation and fatty degeneration with thrombosis of the left ventricle. He discussed Trousseau's theory of cardiac paralysis in diphtheria, and regards his two cases as proof that diphtheria is an infectious disease with general blood-poisoning and injury to vital organs. He attributed the collapse to a cardiac paralysis dependent on the myocardial injury.

Rosenbach²⁷ strengthened greatly the evidence for the existence of a "myocarditis diphtheritica." In 4 cases of diphtheria, he found a myocarditis similar to that seen in other infections. There was marked waxy degeneration and necrosis of the heart muscle, in all respect resembling that seen in the striped muscles in typhoid fever, as described by Zenker, Hayem and others. Some fibers showed granular degeneration. The vacuolation, granular and waxy changes were most marked near the endocardium. In one case, marked fatty degeneration of the fibers, with less waxy degeneration than in the others, was present. Infiltrations of lymphocytes and larger spindle cells with pale-staining protoplasm and simple nuclei were present between the fibers. The stable cells of the perimysium were enlarged and increased in number. In the areas of degeneration, the muscle nuclei were much altered, irregular in size and form, and showed karyorrhexis. Empty perimysial tubes showed complete destruction of some of the fibers. In the less damaged areas, the muscle nuclei seemed little changed, but showed evidences of division. He regarded the process in all 4 cases as an infectious myocarditis, but was unable to demonstrate any micro-organisms in the lesions.

Birch-Hirschfeld²⁸ also described similar parenchymatous and interstitial changes in the hearts in cases of sudden death after diphtheria. He regarded the interstitial changes as primary and the muscle degeneration as secondary to these. Guttman²⁹ observed parenchymatous degenerative lesions in the myocardium in cases of diphtheria as well as in other infectious diseases. He saw no inflammatory reaction. In the same year, Leyden³⁰ made a most important contribution to the theory of an infectious diphtheric myocarditis, based on a study of 3 cases showing a typical acute myocarditis characterized by dilatation and thinning of the wall of the left ventricle with thrombus formation, soft and friable heart muscle, hemorrhagic extravasations, fatty degeneration and atrophy of muscle fibers and intermuscular proliferation. Leyden discarded the older theories of cardiac paralysis and infectious endocarditis, and interpreted his findings as proof of the existence of a typical infectious diphtheria-myocarditis. He considered this to be primarily interstitial, the muscle changes not primary and not proportionate to the inflammatory reaction. He noted further the occurrence of changes in the pharyngeal muscles similar to those found in the heart. He emphasized strongly his conviction that myocarditis is the essential cause of cardiac paralysis in diphtheria.

The clinical entity of diphtheria myocarditis was now established, and observations began to be reported in which the diagnosis was made before death and

²⁶ Arch. f. Heilk., 1873, 14, p. 61.

²⁷ Arch. f. path. Anat., 1877, 70, p. 352.

²⁸ Jahrb. d. Gesellsch. f. Natur. u. Heilkunde, 1878-9, p. 26.

²⁹ Ztschr. f. klin. Med., 1882, 6, p. 144.

³⁰ Ibid., 1882, 4, p. 334.

confirmed by necropsy. Huguenin's article³¹ may be taken as an example. In one case of diphtheria with great cardiac weakness in a 25 year old man, the heart at necropsy showed great dilatation, and on microscopic examination cellular infiltrations of the intermuscular tissue. The walls of the vessels were rich in cells, and many showed endothelial proliferation (endartérite proliférante). The muscle nuclei were much swollen and showed division; the majority of the fibers presented a granular degeneration in varying degrees, a few showed hyaline change. He describes "corps myoplastiques" similar to those seen in myositis by Hayem, undoubtedly regeneration forms. In a second case, a 19-year old girl dying of cardiac syncope on the 15th day of the disease after having shown marked heart weakness for a week, the same changes were found in the heart at necropsy and on microscopic study. No endocarditis was found in either case. He regarded the muscle changes as the essential cause of the cardiac symptoms.

Only a few dissenting reports appeared in the literature. Gombault³² and Cornil and Babes³³ in the same year found no changes in the heart muscle in fatal cases. The latter observers state that the myocardium only rarely shows some fatty degeneration, and that an endocarditis is a still rarer complication of diphtheria. Babes³⁴ also in his investigations on experimental diphtheria states that the thoracic organs show no striking changes, but he does not mention the heart specifically.

As the conception of diphtheria myocarditis became accepted, and with the increase in observations confirming the occurrence, a discussion arose as to the cause and character of the muscle changes. The pathologic changes in the myocardium were variously interpreted as a "parenchymatous" or an "interstitial" myocarditis, and there were many differences of opinion as to the relative importance of granular, waxy and fatty changes in the heart muscle fibers, and also as to the character and significance of the interstitial cellular infiltrations. Two other theories began to receive attention, one of primary damage to the coronary vessels, the other of primary injury to the cardiac nerves and ganglia.

Schemm,³⁵ basing his views on the pathologic study of the hearts in 13 cases of diphtheria, concluded that the changes found correspond to the condition described by Orth as a "myocarditis parenchymatosa seu degenerativa." His cases presented varying degrees of fatty and granular degeneration of the muscle fibers, with slight hyaline change and atrophy, swelling and increase of the muscle nuclei, with interfibrillar cellular infiltrations and proliferation. He noted a direct relationship between the severity of these changes and the duration of the disease. Hochhaus³⁶ noted an interstitial myositis of the myocardium as well as of the pharyngeal and laryngeal muscles. He concluded that the course of diphtheric paralysis may be peripheral or central, either in the muscles and thence extending into the cord, or the reverse. The poison circulating in the blood acts on both muscles and nerves. In the same year, the alterations of the myocardium in diphtheria were studied by Savigné,³⁷ Rabot and Philippe³⁸ and Romberg.³⁹ Rabot and Philippe found hyaline and fatty changes in the heart muscle with interstitial round-cell infiltrations, and considered the process essentially interstitial. Romberg studied the myocardial changes in typhoid, scarlet fever and diphtheria. In

³¹ Rev. de méd., 1888, 6, p. 790, 995.

³² Cited by Scagliosi.

³³ Les Bactéries, 1886.

³⁴ Arch. f. path. Anat., 1890, 119, p. 460.

³⁵ Ibid., 1890, 121, p. 235.

³⁶ Ibid., 1891, 124, p. 226.

³⁷ Des alterations de la myocardi dans le diphthérie, 1891.

³⁸ Arch. d. med. exper. et l'anat. path., 1891, 3, p. 646.

³⁹ Deutsch. Arch. f. klin. Med., 1891, 48, p. 369.

all of these, there occur parenchymatous degenerative changes (granular, hyaline or fatty degeneration) and a true interstitial myocarditis characterized by small cell infiltrations. In 8 cases examined, all showed a more or less marked myocarditis with associated periendocarditis and endocarditis, periarteritis and perineuritis in the majority of cases. Acute and chronic stages may be observed according to the stage of the infection. He regarded the parenchymatous changes as secondary to the myocarditis. He was unable to obtain diphtheria bacilli from the myocardial lesions; and, therefore, regarded the cardiac lesions to be, not the direct result of infection, but the result of the action of the specific toxalbumin.

Romberg's theory of a toxic myocarditis was confirmed in the next year by Welch and Flexner,⁴⁰ who showed that guinea-pigs inoculated subcutaneously in the belly wall with sterile cultures of diphtheria bacilli presented fatty changes in the myocardium. This experimental work was confirmed in 1894 by Comba⁴¹ and Henriquez and Hallin.⁴² The former found that animals inoculated either with cultures of the Löffler organism or with sterile filtrates of the same, developed the same cardiac changes as those found in human cases of the infection, these alterations involving both muscle fibers, interstitial tissue and blood vessels, but most marked in the muscle. He, however, could find no direct relationship between the parenchymatous and interstitial changes. Henriquez and Hallin found that the injection of the soluble products of diphtheria cultures led to the production of thickenings of the left ventricle.

With the establishment of the proof of the existence of diphtheria toxin and its action on the myocardium, the pathologic studies of the heart in diphtheria were greatly stimulated along the lines of the mechanism of this toxic action. Diverging views developed, some writers holding that the cardiac injury was primarily vascular, others that the vagus and cardiac ganglions were primarily involved, while others continued the discussion of a primary myocarditis, either parenchymatous or interstitial. Hesse⁴³ also made an experimental study of the results of the injection of diphtheria cultures and studied also the hearts of 29 children dying of diphtheria. He found especial changes in the vessel walls leading to the migration of leukocytes and to the development of an interstitial myocarditis. Neither the parenchymatous change nor an interstitial myocarditis was a constant finding; the only change present in all cases was that found in the small branches of the coronaries in the form of dilatation, swelling and proliferation of the vessel wall, perivascular hemorrhage and cell migration. He did not, however, consider these vascular changes sufficient to explain the cardiac death of diphtheria. The paralysis of the heart he considered the result of the direct action of the diphtheria toxin on the heart muscle. Hesse's view, in fact, represented a revival of those held by Hayem⁴⁴ and Martin,⁴⁵ both of whom looked on a coronary endarteritis as the essential cardiac injury of diphtheria.

In the same year, Veronese⁴⁶ brought up again the theory of a primary vagus injury, which had been advanced by Gulat.⁴⁷ He considered the pathologic histology of the heart in diphtheria to vary in different cases, but to consist essentially of a parenchymatous degeneration (fatty) of the muscle with interstitial proliferation, associated with the occurrence of degenerative changes in the vagi, heart

⁴⁰ Bull. Johns Hopkins Hosp., 1891, 2, p. 25; 1892, 3, p. 17.

⁴¹ Lo sperimentale, 1894. Cited by Scagliosi.⁵⁴

⁴² Le Progrès Médical, 1894, 20, p. 463.

⁴³ Jahrb. f. Kinderh., 1893, 36, p. 19.

⁴⁴ Arch. de physiol. norm. et path., 1870, 3, p. 81, 269, 422, 475, 569.

⁴⁵ Rev. de méd., 1881, p. 383; 1883, p. 103.

⁴⁶ Wien. klin. Wchnschr., 1893, 6, p. 403.

⁴⁷ Essai sur la paralysie diphtheritique du nerf pneumogastrique, 1881.

nerves and ganglions, and abdominal sympathetic. Vincent⁴⁸ also regarded injury to the vagi as the essential cause of the cardiac disturbances of diphtheria. On the other side of this question was placed the evidence of Leyden,⁴⁹ Unruh,⁴⁹ Huguenin and Hochhaus, who had been unable to demonstrate any changes in the vagi or cardiac ganglions in the course of the studies made by them.

The question of a myocarditis was taken up again by Romberg,⁵⁰ who found fatty, waxy and albuminoid degeneration of the cardiac muscle, with segmentation of the fibers (Renaut's myocardite segmentaire). In his general resumé of the treatment of diphtheria by antitoxin, Sydney Martin⁵¹ mentioned fatty degeneration of the heart muscle as the only cardiac lesion. Krehl⁵² found vascular changes but none in the cardiac ganglions. Schamschin⁵³ studied 12 necropsy cases, and gave a complete description of the myocardial changes in different stages of the disease. Two of his cases had been treated with antitoxin. The most constant and marked lesions were fatty and waxy degeneration of the heart muscle. The fatty changes were usually focal, rarely diffuse. He gives a good description of the waxy necrosis of the heart fibers and of the reparative interstitial processes, noting division of the muscle nuclei and the occurrence of cells analogous to those described by Hayem as "corps myoplastiques," but was unable to decide as to any regeneration of the heart muscle. He found no changes in the blood vessels and none in the cardiac nerves and ganglions. He concluded that the disturbances of the heart in diphtheria are due wholly to the structural changes in the heart muscle resulting from the action of the toxin on the muscle. The presence of the toxin in the muscle alone is not sufficient to cause paralysis; the latter will occur only when the toxin causes deep nutritional and structural changes in the muscle.

Scagliosi,⁵⁴ on the ground of 6 cases studied at necropsy, came to the conclusion that the toxin of diphtheria first affects the wall of the blood vessels, thereby more readily reaching the muscle in which it causes parenchymatous changes. He would classify the process as a true "myocarditis parenchymatosa." Mollard and Regaut⁵⁵ noted the occurrence of hyaline degeneration of the muscle of the heart in diphtheria. Flexner,⁵⁶ in the same year, mentions fatty degeneration of the myocardium as a result of the action of diphtheria-toxin. Hibbard⁵⁷ reverts to older theories, saying that the cause of death in diphtheria is usually cardiac thrombosis, dilatation or paralysis, produced most probably by the diphtheria toxin. This was based on a clinical study of 800 consecutive cases of diphtheria. No microscopic examinations were made. Hallwachs⁵⁸ made a careful histologic study of the heart in diphtheria, agreeing with Romberg that the cardiac complications in diphtheria are the result primarily of anatomic lesions in the myocardium. These consist of an intense and widespread degeneration (fatty and granular) and waxy necrosis of the cardiac muscle, followed by proliferative and reparative changes. Fibrosis may result, even within 4 to 7 weeks. Following the parenchymatous injury, usually in the 2d week, a progressive myocarditis begins which may lead to death in a few days or may progress over many weeks.

⁴⁸ Arch. de méd. exper. et d'anat. path., 1894, 6, p. 513.

⁴⁹ Jahrb. f. Kinderh., 1883, 20, p. 1.

⁵⁰ Deutsch. Arch. f. klin. Med., 1894, 53, p. 141.

⁵¹ Brit. Med. Jour., 1895, 2, p. 287.

⁵² Deutsch. Arch. f. klin. Med., 1893, 51, p. 416.

⁵³ Beitr. z. path. Anat., 1895, 18, p. 47.

⁵⁴ Arch. f. path. Anat., 1896, 146, p. 115.

⁵⁵ Ann. de l'Inst. Pasteur, 1897, 11, p. 97; Jour. Physiol., 1899, 1, p. 875.

⁵⁶ Bull. Johns Hopkins Hosp., 1897, 6, p. 259.

⁵⁷ Med. and Surg. Rep. City Hosp., Boston, 1898, p. 33.

⁵⁸ Deutsch. Arch. f. klin. Med., 1899, 64, p. 770.

In favorable cases healing may take place, and fibrosis may result. He found no changes in the cardiac ganglions, and only slight perineuritis. His conception of the process is essentially that of Romberg, an infectious myocarditis. Ribbert,⁵⁹ on the grounds of 4 cases studied, representing respectively stages of 10 days, 16 days, 4 weeks and 8 weeks in the duration of the infection, found fatty degeneration always present, but he regarded it of less importance than the associated waxy necrosis. He states that the hyaline muscle stains reddish with van Gieson's stain, while the normal fibers remain colorless. Following the hyaline necrosis, there develops a reparative process which is wholly secondary to the primary muscle injury. The process, therefore, cannot be considered as a true primary interstitial myocarditis. He makes no mention of muscle regeneration. Loewenthal,⁶⁰ using Ribbert's technic, examined the hearts of 34 cases of diphtheria with reference to the occurrence of waxy necrosis, and did not find it in any case. In 4 of the cases cellular infiltrations were present in the myocardium.

By the close of the century, the knowledge attained of the myocardial lesions in diphtheria was well summed up by Baginsky.⁶¹ He states that the changes produced in the heart in diphtheria are manifold, and are especially marked in the cases in which death takes place some time after the attack, the general symptoms being those of cardiac inadequacy. On the whole, the cardiac changes are found early in the affection, but may be missed in children who die quickly with severe septic symptoms; in these, the only lesions found in the heart are subepicardial hemorrhages. The most common change in cases of longer duration is a fatty degeneration of the muscle fibers, which may progress to a complete destruction of the muscle substance, with a corresponding degeneration of the muscle nuclei. Associated with the fatty change is a colloid or waxy-transformation of the muscle substance, with complete loss of striation. Stretches of muscle showing striation alternate with those showing waxy and fatty degeneration. In the latter areas, the connective tissue also may be hyaline. In the neighborhood of the areas of greatest alteration of the fibers, there is an interstitial round-cell infiltration and an increase of muscle nuclei. He regarded the appearances of fragmentation of the muscle as representing a pathologic and not a postmortem change. Marked congestion of the capillaries and hemorrhagic extravasations form also a feature of the diphtheria myocarditis.

The early years of the new century were prolific in writings bearing on this subject. Among the most important is the study of the pathologic findings in 220 fatal cases of diphtheria by Councilman, Mallory and Pearce.⁶² They found that myocardial degeneration is one of the most common lesions of diphtheria. Fatty degeneration was the most frequent, accompanying or preceding vacuolation or hyaline change, fragmentation or fractional degeneration of the fibers. In the severe cases of short duration, simple fatty degeneration alone was present; in the more prolonged cases, the cardiac degeneration may be so extensive as to account for the symptoms. They attempted to distinguish two kinds of interstitial changes: one consisting of focal collections of plasma and lymphoid cells accompanied by degeneration of the muscle but not dependent on it; while another consisted of secondary proliferative changes that may lead to extensive formation of connective tissue, and thus be responsible for some cases of fibroid heart. They found thrombosis a not uncommon complication and considered it due to a primary necrosis of the endocardium. They thought that lesions of the coronary vessels played but little part in the cardiac pathology

⁵⁹ Mitt. a. d. Grenzgeb. d. Med. u. Chir., 1899-1900, 5, p. 1.

⁶⁰ Centralbl. f. allg. Path. u. path. Anat., 1900, 11, p. 612.

⁶¹ Nothnagel System, 1899, 2, p. 121.

⁶² Jour. Boston Soc. Med. Sc., 1901, 1, p. 137.

and symptomatology. Deguy and Weill⁶³ took up again the question of cardiac thrombosis in diphtheria, and decided that the coagula seen in diphtheria were either agonal or postmortem, or were due to secondary infection with cocci. Eppinger⁶⁴ described the occurrence in the heart in diphtheria of a diffuse edema with marked vacuolation and liquefaction of the heart muscle which he termed "myolysis cordis diphtheria toxica." He regarded this as the essential lesion of the postdiphtheric paralysis of the heart resulting from the action of the toxin on the vasomotor system. He returns to the theory of paralysis of the vasomotor centers in the medulla held by Romberg, Pässler, Brühns, Müller, Rolly, Girard, and others, as the essential cause of early death in diphtheria. Thorel and other writers were unable to confirm Eppinger's findings, and it has been generally assumed that he was dealing with some form of artefact.

Cowan⁶⁵ discussed all forms of pathologic changes occurring in the heart muscle in the acute infections. He quotes various writers who had observed the occurrence of fatty degeneration in the heart in diphtheria, but concludes that cloudy swelling is the most common change in the cardiac muscle in acute diseases, that fatty degeneration is not rare, while hyaline degeneration is an occasional change and rarely extensive. Förster,⁶⁶ in a clinical article emphasizing the importance of diphtheric myocarditis, says that every infectious disease has its specific form of myocarditis. The intensity of the clinical symptoms of diphtheria myocarditis varies greatly in different children. In the same year, Schmaltz⁶⁷ reviewed the literature of the cardiac changes in diphtheria up to 1900. He summed these up as consisting essentially of a more or less marked fatty, albuminoid or waxy degeneration, often necrosis of whole layers of heart muscle with changes in protoplasm and nuclei, with round cell infiltration and proliferation of the connective tissue. These changes are usually most marked at the base of the heart or near the apex. Extravasations, and some degree of endocarditis or pericarditis may be associated with the myocardial changes. Escherich⁶⁸ reported observations on cerebral embolism associated with postdiphtheric thrombi on the mitral valves; and called attention to the possibility of confusing paralysis due to cerebral lesions with postdiphtheric paralysis.

The problem of the diphtheria heart-death was now to be attacked from another point of view, that of pathologic physiology rather than of pathologic anatomy and histology. The new theories of cardiac rate and rhythm based on the anatomic foundation of sino-auricular and auriculoventricular nodes and the conducting bundle naturally led clinicians to electrocardiographic studies of the heart in diphtheria. These studies soon demonstrated that symptoms interpreted in the electrocardiogram as showing disturbances of the conducting system were common in diphtheria. Fleming and Kennedy⁶⁹ reported a case of complete heart block in diphtheria with necropsy study of the heart. A definite myocarditis with inflammatory foci in the conducting bundle was found. The auriculoventricular node and the first part of the auriculoventricular bundle were involved in the inflammatory process. Both vagi were examined and found to be normal. Price and Mackenzie⁷⁰ studied a heart from a case of diphtheria showing auricular fibrillation and heart block and found an extensive myocarditis.

⁶³ Arch. de méd. expér. et d'anat. path., 1902, 14, p. 427.

⁶⁴ Deutsch. med. Wchnschr., 1903, 29, p. 288.

⁶⁵ Jour. Path. and Bacteriol., 1903-4, 9, p. 86.

⁶⁶ Deutsch. Arch. f. klin. Med., 1905, 85, p. 35.

⁶⁷ Ibid., p. 10; Jahrb. f. Kinderh., 1897, 45-46, p. 89.

⁶⁸ Wien. klin. Wchnschr., 1907, 20, p. 179.

⁶⁹ Heart, 1910, 2, p. 77.

⁷⁰ Ibid., 1911, 3, p. 233.

particularly marked in the ventricles, but not involving the sino-auricular node or bundle. No changes were noted in the nerves or ganglions. They attributed the cardiac paralysis to the widespread myocarditis.

Coghlan⁷¹ came to the conclusion that the cause of all of the circulatory disturbances in diphtheria is to be found in the degenerative changes produced by the direct action of the toxin on the heart muscle. The occurrence of extrasystoles is to be taken as important evidence that the heart wall is involved; and a reduplication of the first sound followed by the accentuation of the second is a sign of advanced myocardial disease. In the same year, Rohmer⁷² made electrocardiographic studies of the cardiac disturbances in diphtheria with reference to their relation to the conducting system. He concluded that the diphtheria toxin had no especial affinity for the latter. Likewise Tanaka⁷³ thought that lesions of the conducting system were rarely the cause of death. In 14 out of 15 patients dying in cardiac syncope, he found fatty degeneration, and in 6 cases an interstitial inflammation with polymorphonuclear, mononuclear and eosinophile infiltration. Abramow,⁷⁴ also in the same year, advanced the idea that subacute death in diphtheria is due to regressive changes in the heart muscle due to lack of epinephrin.

Hecht⁷⁵ found fatty degeneration, cloudy swelling and interstitial round-cell infiltration to be the chief anatomic changes in the heart in diphtheria, and thought that the cardiac disturbances were to be referred to these. Anitschkow⁷⁶ described in detail the changes produced in the myocardium of rabbits by the diphtheria toxin. He found the same degenerative changes, granular and fatty, and hyaline necrosis ("homogenisation") noted by so many observers in both experimental work and in human diphtheria. He added, however, a more complete description of the reactive and reparative changes associated with or following such an injury than had up to that time appeared in the literature. He found distinct evidence of regeneration of the heart muscle in the form of "myocytes," cells of myogenic origin arising from the injured portion of the muscle or from preexisting myogenic elements in the neighboring uninjured portions. He considered the interstitial inflammatory changes as purely a secondary reparative process following the primary muscle lesion. The dead portions of the muscle act as a foreign substance leading to the production of foreign-body giant cells about it. Heller⁷⁷ made a study of 30 diphtheria hearts. He found granular degeneration to be much more common than fatty change; waxy degeneration was also common, particularly in the later stages; simple necrosis of portions of the muscle fibers was also seen. He also saw much evidence of regeneration of the heart muscle; in fact, his paper is chiefly an argument in favor of such regeneration. He describes fully the cell character of the interstitial reaction and the regenerative process. Of especial interest is his description of perimysial tubes filled with detritus of dead muscle substance, leukocytes, myogenic cells or new protoplasmic bands arising from a splitting of the ends of the living muscle fibers.

Vulfius⁷⁸ noted the occurrence of groups of eosinophile cells in the heart muscle of diphtheria patients, these cells appearing on about the 7th day of the

⁷¹ Brit. Med. Jour., 1912, 1, p. 534.

⁷² Ztschr. f. exper. Path. u. Therap., 1912, 11, p. 426.

⁷³ Arch. f. path. Anat., 1912, 207, p. 115.

⁷⁴ Ztschr. f. Immunitäts., 1912, 15, p. 12.

⁷⁵ Ergebn. d. inn. Med. u. Kinderh., 1913, 2, pp. 324, 413.

⁷⁶ Beitr. z. path. Anat. u. z. allg. Path., 1913, 56, p. 379; Arch. f. path. Anat., 1913, 211, p. 176.

⁷⁷ Beitr. z. path. Anat. u. z. allg. Path., 1913-14, 57, p. 223.

⁷⁸ Vratsh. Gaz., 1914.

disease. Blacher⁷⁹ decided that a gallop rhythm and extrasystoles cannot be taken as a direct evidence of the degree of anatomic change in the myocardium, although they often occur in association with a myocarditis. Hume and Clegg⁸⁰ reported observations on the occurrence in diphtheria of auricular and ventricular premature beats, paroxysmal tachycardia, auricular flutter and fibrillation, heart block, nodal premature beats and reversal of nodal beats. The pathologic study showed marked fatty degeneration of the ventricular walls, with extreme engorgement of the smallest capillaries and scattered hemorrhages. The sino-auricular node showed increased vascularity and hemorrhage with lymphocyte infiltration. The auriculoventricular node and bundle of His showed engorgement and dilatation of the capillaries.

As early as 1899, Brodie⁸¹ had advanced the view that the chief cause of diphtheria heart-death within the first 48 hours after the onset of the infection is "failure of the blood-vessels and consequent fall in blood-pressure." In 1914, MacCallum⁸² made an experimental study of the action of diphtheria toxin on the dog's heart, and found that the actual work of the diphtheria poisoned heart was as good as that of the normal heart. He concluded that death occurring during the height of an attack of diphtheria is not necessarily the exclusive result of a direct injury to the heart.

During the next 4 years, practically nothing of any importance appeared concerning the heart in diphtheria. Mackenzie⁸³ stated in his textbook that in diphtheria, more than in any other disease, there is a tendency to fatal syncope, the mechanism of which he is unable to understand. The heart muscle may be the seat of profound changes, the symptoms somewhat resembling those of rheumatic fever. The cardiac complications are so varied that danger may arise in several ways. From 1918-20, Aviragnet, Lutembacher and Soudier,⁸⁴ in a series of papers emphasized their idea that the diphtheria toxin has an especial affinity for the bundle of His. In their first article (1918), they describe in detail the microscopic features of the toxic myocarditis of diphtheria. The parenchymatous lesions are marked alteration in the majority of the myocardial fibers, with atrophy, tumefaction, vacuolation, loss of striation, fragmentation, and a great variety of nuclear changes even to complete karyolysis. No fatty changes were seen. The interstitial lesions were characterized by edema, leukocyte and mononuclear infiltrations, with the presence of fibroblasts and myoblastic bodies. These changes were especially marked in the region of the bundle of His, but extended throughout the entire myocardium. Thrombosis and endocarditis are purely secondary and incidental. Any severe cardiac disturbance in diphtheria must be ascribed to the action of the toxin on the muscle elements.

Nuzum⁸⁵ noted the occurrence of an eosinophile infiltration in the myocardium in 7 of 29 cases of diphtheria. He was unable to find anything like it in many cases of death from other infectious diseases. It will be remembered that Vulfius in 1914, made a similar observation. These are apparently the only recorded observations in which the myocardial infiltrations showed any eosinophile preponderance, although Aschoff and Kaufmann both show eosinophile infiltration in their figures illustrating diphtheria myocarditis.

⁷⁹ *Jahrb. f. Kinderh.*, 1914, 29, p. 166.

⁸⁰ *Quart. Jour. Med.*, 1914-15, 8, p. 1.

⁸¹ *Brit. Med. Jour.*, 1899, 2, p. 128.

⁸² *Am. Jour. Med. Sc.*, 1914, 147, p. 37.

⁸³ *Diseases of the Heart*, 1918.

⁸⁴ *Arch. d. mal du cœur*, 1918, 11, p. 241; *Jour. méd. franc.*, 1920, 9, p. 19; *Arch. d. mal. du cœur*, 1920, 13, p. 1.

⁸⁵ *Jour. Am. Med. Assn.*, 1919, 73, p. 1925.

McCulloch⁸⁶ made electrocardiographic studies on 19 of a group of 80 cases of diphtheria. In these 19, there were evidences of cardiac disturbance of varying degree. In profound cases with interference of the cardiac conducting mechanism, the outcome was usually fatal. Recovery in any case, seems to depend on the ability of the heart to preserve a cardiac reserve sufficient for the need of the patient. Cardiac failure results when this reserve is used up. Severe functional changes result, tending to a disturbance in the cardiac mechanism. The occurrence of diphtheria myocarditis is beyond our control. He considers that it has a definite position among those factors that lead to chronic heart disease later in life. Farr⁸⁷ also described the clinical forms of cardiac disturbances in diphtheria, showing that practically all forms of disturbances of rate and rhythm occur in this infection. He holds that the cardiac complications involve chiefly the myocardium, for which the diphtheria toxin has a peculiar affinity. The cardiac nerves and blood vessels and the vasomotor center may also be involved. Rupe⁸⁸ thought that lesions of the myocardium may cause no clinical symptoms, but that the slightest lesion of the bundle of His will produce clinical manifestations of disturbances of conduction.

Richardson,⁸⁹ in an analysis of 100 cases of diphtheria, states that 40% showed myocarditis. Holt⁹⁰ thinks that the explanation of heart failure in or after diphtheria is not always the same. In the early cases, it may be due to coronary thrombosis associated with degenerative changes in the muscle. Later in the disease, it is probably always the result of a true toxic myocarditis which is more important than any lesion of the cardiac nerves. Allen⁹¹ explained a case of complete heart block in a case of diphtheria as being due to poisoning of the auriculoventricular bundle. Friedeman⁹² formerly held the view that the muscle changes of the heart in diphtheria were secondary to a loss of tone following nerve paralysis. As the work of Eckstein has shown that muscle tone is primarily myogenic and not neurogenic, he has abandoned his original position. The direct injection of epinephrin into a dilated heart in diphtheria caused it to contract, and the pulse was lowered, but later it dilated again, and the patient died.

Bie and Schwensen⁹³ described the effect of digitalis in two cases of diphtheria arrhythmia. One patient recovered; the other died from progressive acute myocarditis which involved the atrioventricular bundle and produced partial heart block. Schwensen⁹⁴ reviewed the symptomatology of the cardiac disturbances of diphtheria. He examined 568 cases of diphtheria, of which 118 were severe cases and 8 were fatal. In 17% of the total, 75% of the severe, and in all of the fatal, cases there were signs of myocarditis. He noted the occurrence of two types of rhythm disturbances; one appearing by the 8th day, organic heart block with pallor, syncope, cool skin and early death; the second, appearing late (33rd day) shown by extrasystoles. No death occurred in this stage. In 4 cases of the first type, the histologic examination showed the same involvement of the atrioventricular node and bundle as did the general myocardium. He regards diphtheria as an important cause of cardiac insufficiency later in life. One patient examined 2 years after the attack showed cardiac impairment.

⁸⁶ Am. Jour. Dis. Child., 1920, 20, p. 89.

⁸⁷ Pennsylvania Med. Jour., 1920, 23, p. 633.

⁸⁸ Abstr. Mod. Med., 1920, 2, p. 210.

⁸⁹ Rhode Island Med. Jour., 1920, 3, p. 87.

⁹⁰ Diseases of Infancy and Childhood, 1920, p. 835.

⁹¹ Brit. Med. Jour., 1921, 1, p. 267.

⁹² Deutsch. med. Wchnschr., 1921, 47, p. 1581.

⁹³ Jour. Infect. Dis., 1922, 30, p. 308.

⁹⁴ Ibid., p. 279.

Warthin,⁹⁵ in a preliminary paper, reviews the literature, and presents a study of the cardiac pathology in 16 cases of diphtheria. The present article represents the completed study of this material. Loth⁹⁶ reviews the more recent literature of cardiac pathology in diphtheria with especial reference to the discussion concerning the involvement of the conducting mechanism. She made a pathologic study of the hearts in 19 cases of diphtheria and of the results of the action of diphtheria toxin in 4 guinea-pigs. Her main conclusion was to the effect that while the diphtheria toxin does damage the heart (fatty degeneration), it does not, even in fatal doses resulting in delayed death, bring about a true myocarditis. In this respect, she thinks the findings in experimental animals agree with those in the majority of fatal cases of human diphtheria. In her summary, she states that a large proportion of diphtheria fatalities are due to circulatory failure, resulting probably from injury to the myocardium or its conducting system, although experimental work (MacCallum) points to a serious disturbance in the peripheral vasomotor system. She could demonstrate no specific myocardial lesions in her cases; fat accumulation and cloudy swelling were regularly seen but were not more pronounced than in other acute infections. A definite inflammatory reaction was only exceptionally seen at necropsy.

This review of the literature of diphtheria extending over nearly three-quarters of a century is of interest in showing how the various theories concerning the nature of the cardiac disturbances held at any given time were influenced by the prevailing line of thought of that period. The flux and ebb of scientific theory and opinion as to the essential heart lesion in diphtheria, extending even to the present time, is shown by the last author named, Loth, who, ignoring all of the pathologic evidence of the importance of parenchymatous injury and myocarditis in diphtheria built up by the German school, minimizes the clinical importance of such myocardial injury in favor of a theory of disturbances of the peripheral vasomotor system. The textbooks on medicine and affections of the heart used at the present time show no agreement in their treatment of this subject, and there is no conception of the cardiac condition as a definite entity presenting itself in different aspects according to its severity and duration. Primary injury to the heart muscle in the form of fatty degeneration, cloudy swelling, hyaline degeneration or necrosis, vacuolation and fragmentation of the heart muscle, with or without evidences of inflammatory reaction, repair or regeneration, is regarded by individual authors as the essential lesion of the myocardium or its conducting system, or of both; while others regard the myocardial changes as secondary, and attempt to explain the circulatory failure as the result of disturbances in the sympathetic nerves or ganglions, or in the peripheral vasomotor system. The English and American writers all overemphasize the occurrence of fatty changes

⁹⁵ Tr. Assn. Am. Phys., 1923, 38, p. 70.

⁹⁶ Arch. Int. Med., 1923, 31, p. 637.

in the diphtheria heart as an essential pathologic change, and practically no one of them considers the possibility of a regeneration of the heart muscle. While the clinical studies of the heart in diphtheria are in general agreement as to the symptomatology, the chief difficulty has lain in the interpretation of the pathologic findings and in the correlation of these with the physiologic phenomena.

In the attempt to clear up some of these questions and to give a more definite entity to the cardiac pathology of diphtheria, there is presented here a study of the hearts from 16 necropsy cases of diphtheria.

Fifteen of these cases were furnished by the University Hospital, and the necropsies made in the Pathological Laboratory of the University of Michigan. Of these 15 cases, 1 was from the clinic of internal medicine (Dr. Dock), 5 from otology (Dr. Canfield), 1 from orthopedic surgery (Dr. Abbott), 8 from pediatrics, and all but 1 case coming from the contagious ward (Dr. Cowie). The material of the 16th case was received from Dr. L. Hektoen of Chicago. The cases are considered chronologically.

The method of study was the same for each of the 15 cases necropsied by us. The gross pathology of the heart was noted at necropsy. Blocks were taken from both auricles and ventricles, including portions of the septum and apex. Paraffin imbedding was employed, and the sections stained with hematoxylin and eosin, methylene blue and eosin, and van Gieson's stain. Frozen sections were used for the fat-dyes, sudan III, scharlach R. and Nile-blue sulphate.

CASE 1.—A man, aged 25, a laborer, had been ill for 3 days with sore throat, diagnosed as quinsy. He had marked cardiac dilatation, irregularity and tachycardia. The tonsils and pharynx were covered with diphtheric membrane. The cardiac symptoms grew rapidly worse, and death from cardiac syncope took place a few hours after entrance.

Heart.—Both ventricles and auricles were dilated, flabby, and flattened on a board; the muscle was soft, translucent, and tore easily; it was a pale brownish yellow. There were large agonal clots in both ventricles. There were no changes in the valves or coronary vessels.

Microscopic Examination.—The heart muscle fibers were small and atrophic. There was marked congestion of the blood vessels. Numerous minute hemorrhages were scattered through the myocardium. Perivascular edema was present. There was an increase of leukocytes in the blood vessels. There was slight small cell infiltration around some of the veins (early myocarditis). The muscle showed scattered areas of fatty degenerative infiltration, particularly beneath the endocardium. Many fibers showed a hyaline change with loss of striation and staining power. Other fibers showed marked cloudy swelling, even to necrosis. The majority of nuclei were pyknotic. Some fibers showed advanced Zenker's necrosis with irregular clear spaces in the muscle substance. The endocardium and pericardium were edematous. The pharyngeal striped muscles showed extreme Zenker's necrosis.

Other Organs.—There was extreme congestion, general edema, slight fatty degenerative infiltration of the liver, cloudy swelling of the kidneys, lymphocyte destruction in the spleen with coagulation necrosis of centers of splenic follicles, numerous bone-marrow giant cells in pulmonary and liver capillaries, an extensive diphtheric membrane in the tonsils, palate and pharynx, extending into the larynx. The Klebs-Loeffler bacillus was found.

CASE 2.—A man, aged 40, a farmer, had diphtheria of the pharynx and nose which developed on Feb. 23, 1914. Cultures were positive for the Klebs-Loeffler bacillus; 10,000 units were given. On March 7, 1914, he had a dilated heart, with marked irregularity and tachycardia. The temperature was 103 F. He died in collapse.

Heart.—The pericardial sac contained an increased amount of fluid, of deep yellow color, with numerous white flaky coagula; a smooth and clear epicardium, the vessels markedly congested; the heart twice as large as the cadaver's fist, filled with soft cruor; all cavities of the heart were markedly dilated; the ventricular walls were thin; the muscle soft and brownish.

Microscopic.—The myocardium, especially of the left ventricle, showed extreme Zenker's necrosis with areas of partial absorption of the dead muscle, fibroblastic proliferation and muscle regeneration. The dead muscle looked cooked, waxy and yellowish, and stained poorly with eosin. In areas around the veins, the muscle fibers showed large irregular vacuoles as in advanced Zenker's necrosis. The living muscle in these areas showed myoblastic proliferation, with the development of sarcoplasmic fibrillae extending into the dead muscle. The fat content was slight. Near the proliferating muscle there was lymphocyte and fibroblastic infiltration. The vessels showed extreme congestion and perivascular edema. The microscopic picture is that of a severe parenchymatous myocarditis with beginning inflammation and repair. Sections of the bundle of His showed similar changes, but with more marked edema and hydropic degeneration.

Other Organs.—Findings in the other organs were: marked congestion; cloudy swelling of liver and kidneys; early bronchopneumonia; lipoidosis of intima of aorta; and deficiency of colloid in the thyroid; pharyngeal muscles showed marked Zenker's necrosis with interstitial inflammatory reaction.

CASE 3.—A boy, aged 3, had had diphtheria, becoming ill on May 30, 1914; two weeks after recovery difficulty in speaking and swallowing developed, followed by general muscular weakness. He entered the hospital on July 15, 1914. He could not lift his head on account of weakness of the muscles of the back; he could not support himself on his legs. There was no marked wasting and no toe drop. The palatal reflexes were lost. There was paralysis of the soft palate. The temperature was 99.6 F.; the pulse rate 122; the first sound at the apex was soft. He died suddenly of cardiac failure on July 17, 1914.

Heart.—The heart was greatly enlarged, particularly on the right side, with marked separation of the muscle fibers. Other findings were: slight thickening of the pericardium on the anterior wall of the right ventricle; all cavities dilated, especially the right heart; filled with fluid blood, except the right auricular appendage which contained cruor. The left ventricular wall averaged 14 mm. in thickness; right 4 mm. The valvular orifices were dilated; the flaps and vessels negative. The myocardium was slightly paler than normal and distinctly clouded; it was soft and tore easily.

Microscopic.—The muscle fibers showed a diffuse, slight hyaline change with patches of more marked waxy necrosis. In the fibers showing the slight change, there was a loss of striation and staining affinity for eosin; the fibers were more highly refractive. In the advanced areas, the fibers were yellowish and had lost their nuclei. Areas of marked fatty degenerative infiltration were also present, especially near the endocardium. Some of the papillary muscles showed extreme fatty change. There was no inflammatory reaction and no evidences of repair.

Other Organs.—The patient had a thymicolymphatic constitution; the right testis had not descended. He had had early bronchopneumonia. Passive congestion of all organs, a slight fatty change in the liver and slight cloudy swelling of the kidneys were present.

CASE 4.—A boy, aged 2, was admitted to the hospital on April 23, 1915. He had supposedly aspirated a peanut on the day before. He had paroxysms of coughing, but the peanut was not recovered. These paroxysms were followed by alternate periods of dyspnea and quiet. The patient was cyanotic at one time for a few minutes. Roentgen-ray examination showed bilateral emphysema, but no foreign body was seen. Removal by bronchoscopy was attempted; an apparent foreign body was seen in the trachea, but attempts to remove it were unsuccessful. The thymus was much enlarged, and it was thought that the tracheal obstruction might be due to that. On April 24, 1915, a tracheotomy tube was inserted. A portion of a peanut was removed from the right main bronchus. A slight fever, cyanosis and labored breathing followed. On the following day there were evidences of bronchopneumonia. The temperature was 104.2 F.; on the following day, it was 103.2 F. The patient became cyanotic, and had a pulse rate of 145, an irregular heart dilated on the right and shallow respirations. The clinical diagnosis was membranous bronchitis. He died of cardiac failure on May 5, 1915.

Heart.—This was enlarged, especially on the right side. All cavities were dilated; there were agonal clots. The heart muscle was yellowish, with a fatty shine, translucent, moist, soft and easily torn; the valves and vessels were normal.

Microscopic Examination.—There was an early hyaline change; portions of the fibers were waxy. The sections stained poorly with eosin and showed pyknotic nuclei. Striation was lost. The unaffected portions of the fibers stained well with eosin; the fat content was normal; the vessels congested. There was no inflammatory reaction, and there were no evidences of repair.

Other Organs.—There was an extensive necrotic diphtheric membrane involving the tonsils, pharynx, larynx, trachea and main bronchi; there were aspiration bronchopneumonia with beginning gangrene, marked fatty degenerative infiltration of the liver, and acute passive congestion of all organs. Bacteriologic examination of membrane showed pure cultures of diphtheria bacilli.

CASE 5.—C. H. T., aged 31, entered the hospital on Nov. 24, 1916, with a sore throat, a membrane on both tonsils, positive cultures; 10,000 units were given intravenously and 10,000 units intramuscularly, but without effect; 10,000 units more given. On the 28th, the membrane covered both tonsils, uvula and pharyngeal wall. There was great toxicity, and the patient was delirious. The temperature was 103 F., the pulse rate 142—irregular. Respirations were labored. The patient died on the following day in cardiac syncope.

Heart.—The left side of the heart was in firm rigor; the right side was dilated, the muscle was a pale yellowish brown, especially beneath the endocardium. The valves and muscles were normal.

Microscopic Examination.—There was diffuse cloudy swelling. The fibers had lost their striation and stained more heavily with eosin. The nuclei were pyknotic; there were patches of marked waxy necrosis in which the fibers were waxy, yellowish and took but little or no eosin. The fat content was not increased. The vessels were congested, the interstitial tissue edematous. There was no inflammatory reaction and no evidences of repair.

Other Organs.—There were a diphtheric membrane on the tonsils, palate, pharynx, larynx, trachea and bronchi; multiple hemorrhages and hemorrhagic infarctions of lungs; Zenker's necrosis of the pharyngeal muscles; passive congestion and cloudy swelling of all organs; chronic orchitis; peri-adrenitis, peripancreatitis and pancreatitis, probably syphilitic; colloid goiter and tattoo.

CASE 6.—A girl, aged 2½ years, two weeks before admission had developed difficult respiration and inability to talk, with cyanosis. There was no history of

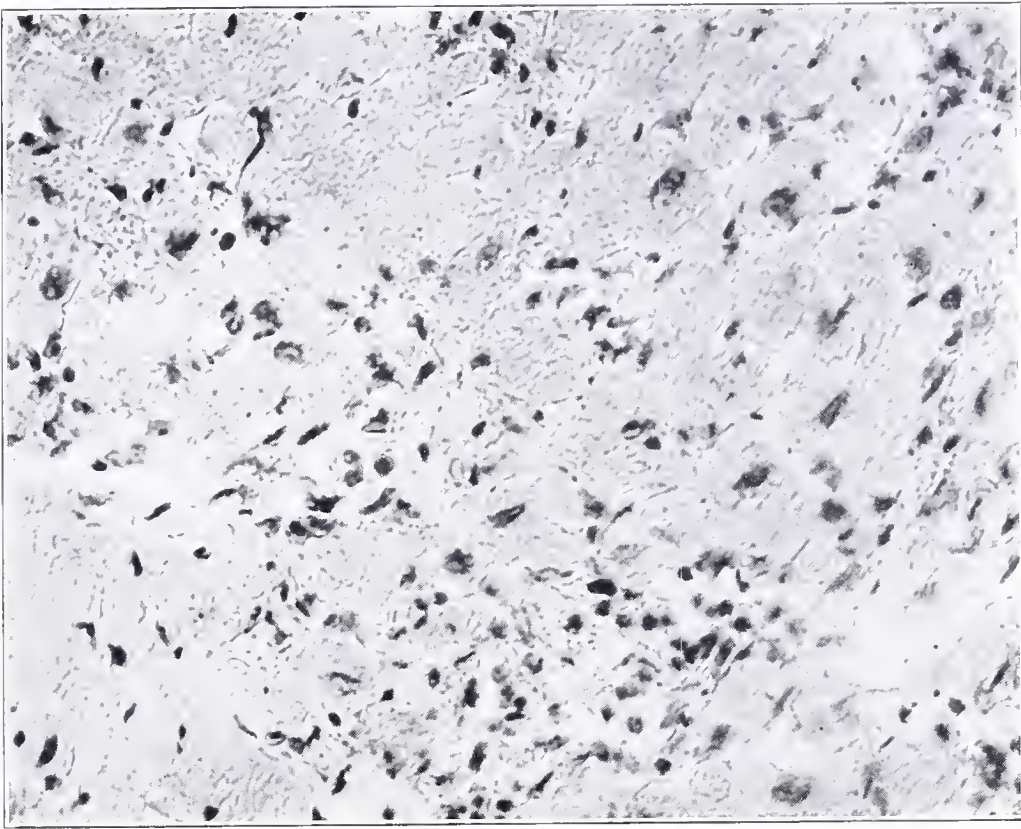


Fig. 1.—Area of myocardium from case 2. Hyaline degeneration and necrosis with beginning repair.

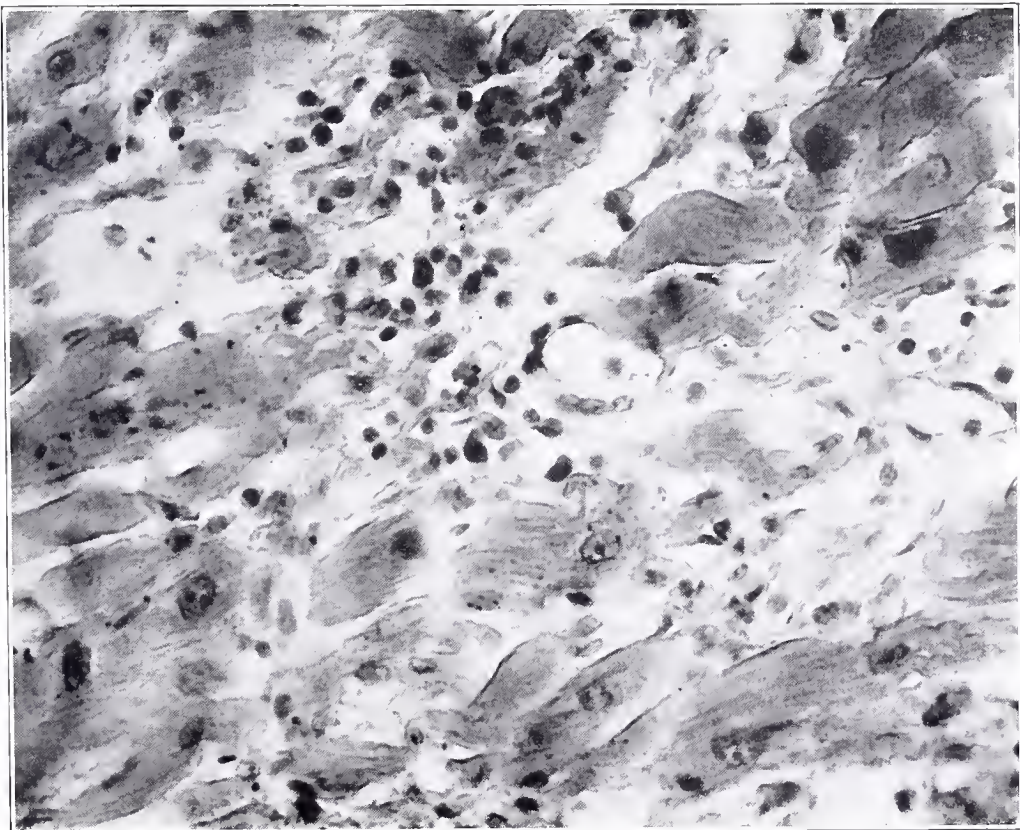


Fig. 2.—Higher power detail of fig. 1. Stage of "interstitial myocarditis;" early repair.

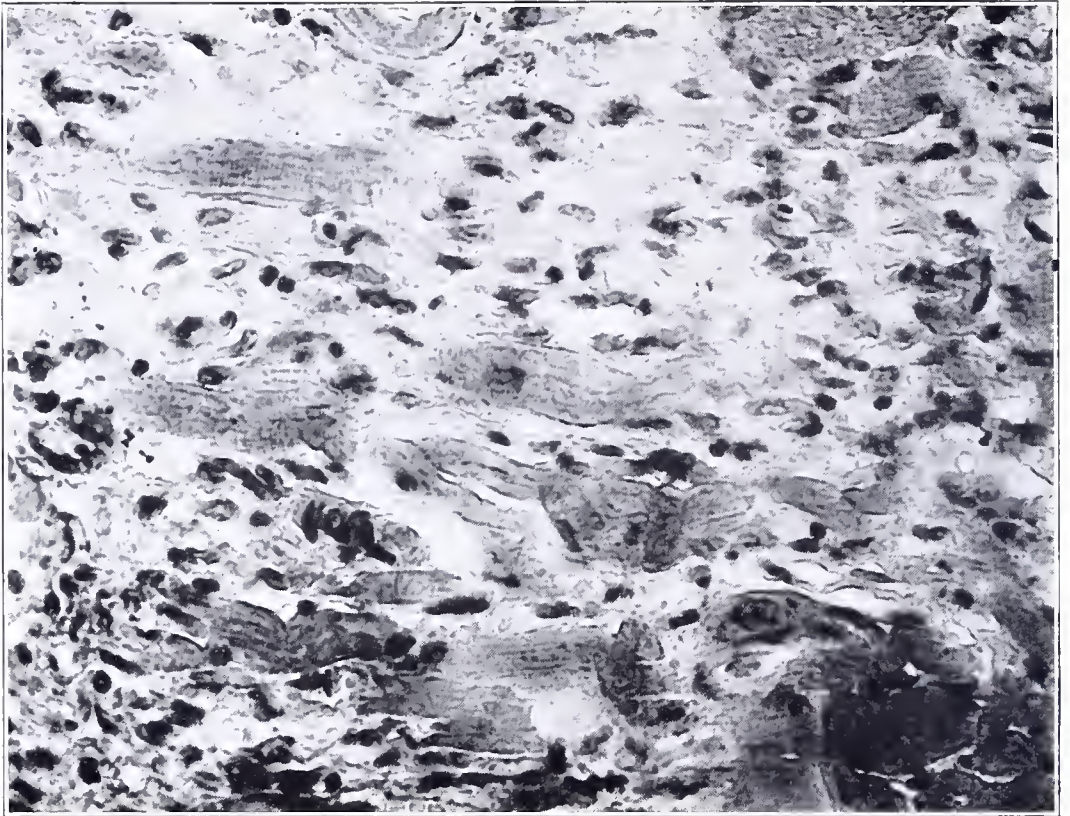


Fig. 3.—Area from myocardium of case 2, showing early muscle regeneration. Numerous myoplasts accompanied by perimysial cells replace the detritus in the collapsed perimysial tubes.

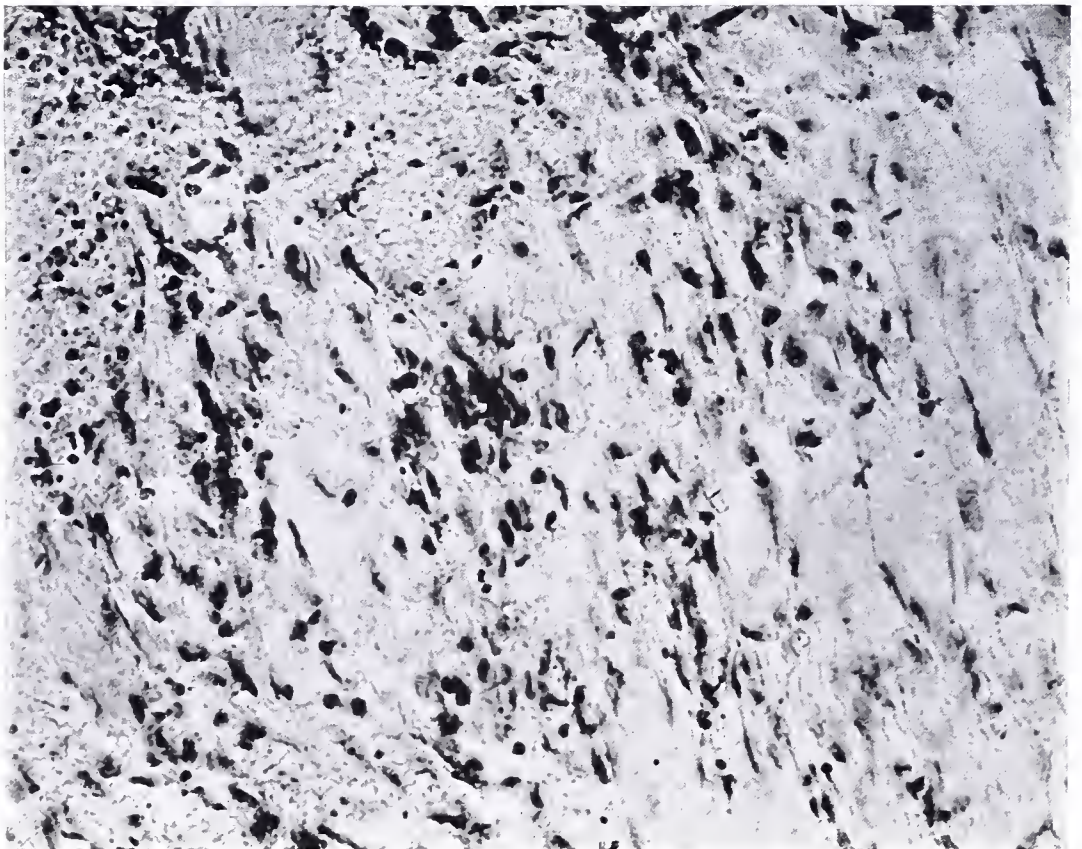


Fig. 4.—Area from same heart as in fig. 3, showing myoplastic cells and protoplasmic bands extending into necrotic heart muscle; muscle regeneration.

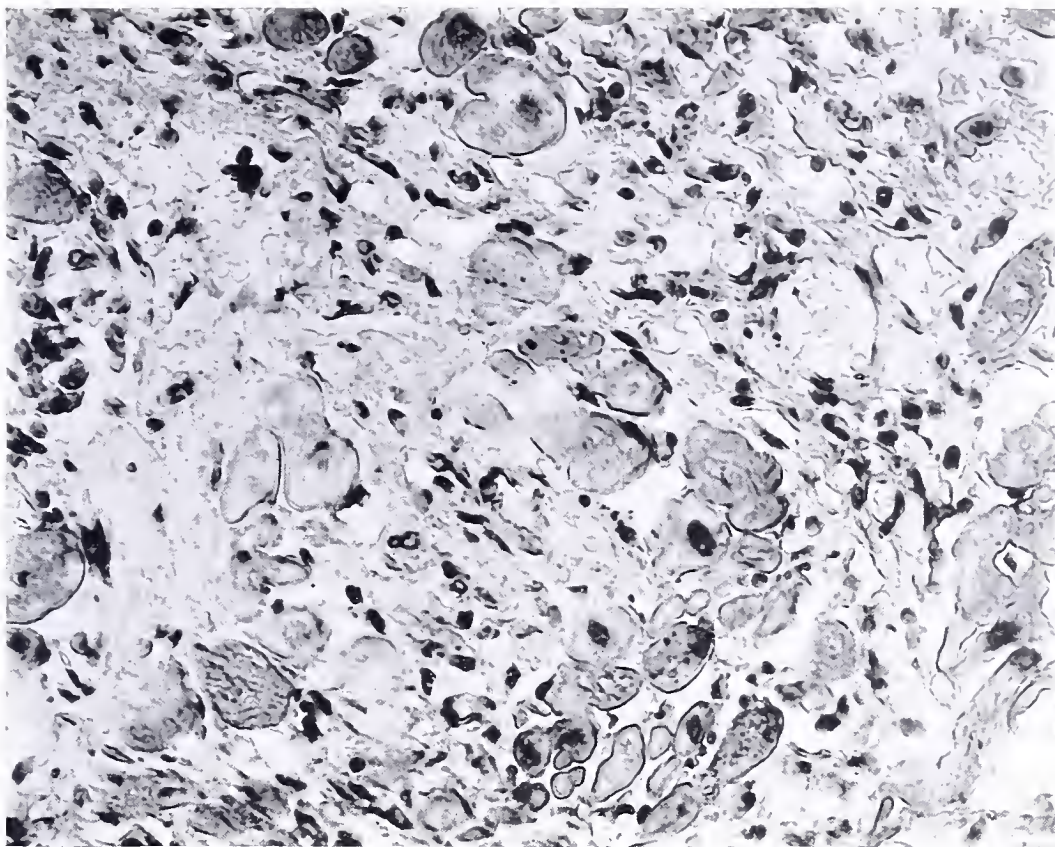


Fig. 5.—Myocardium from case 3. Advanced hyaline necrosis of muscle fibers; beginning formative proliferation.

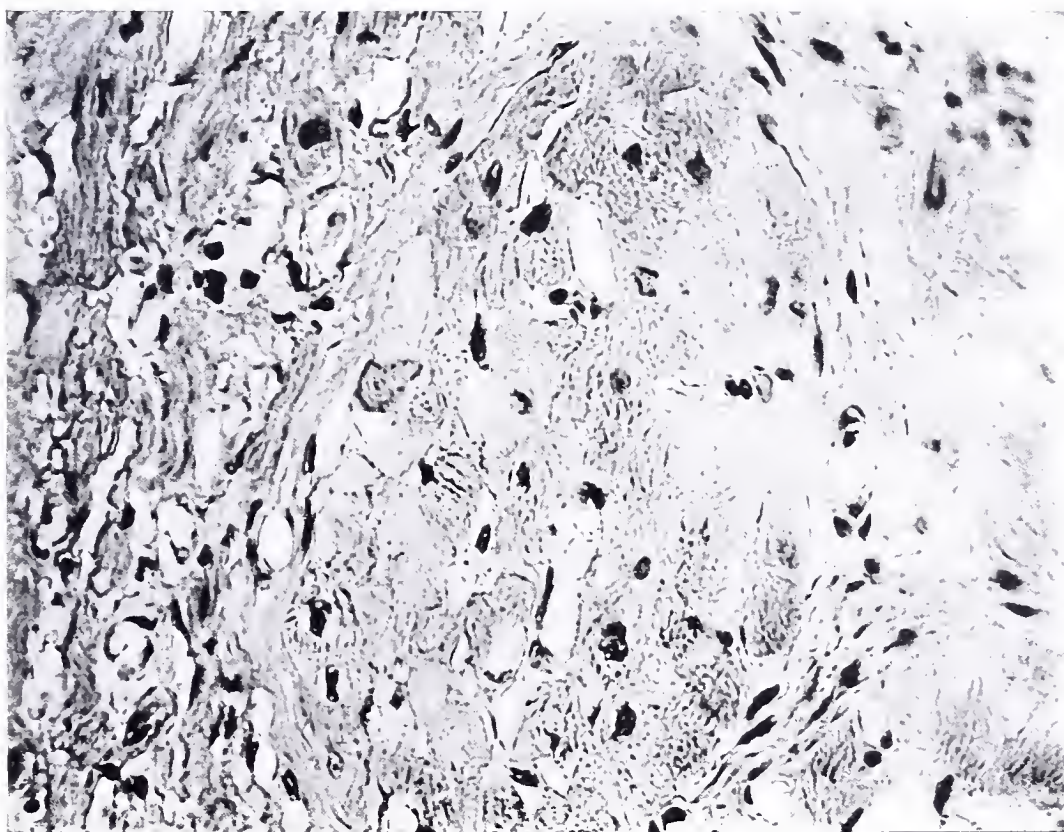


Fig. 6.—Myocardium from case 3, showing portion of His bundle with hyaline change and vacuolation. Neighboring heart muscle shows marked necrosis.

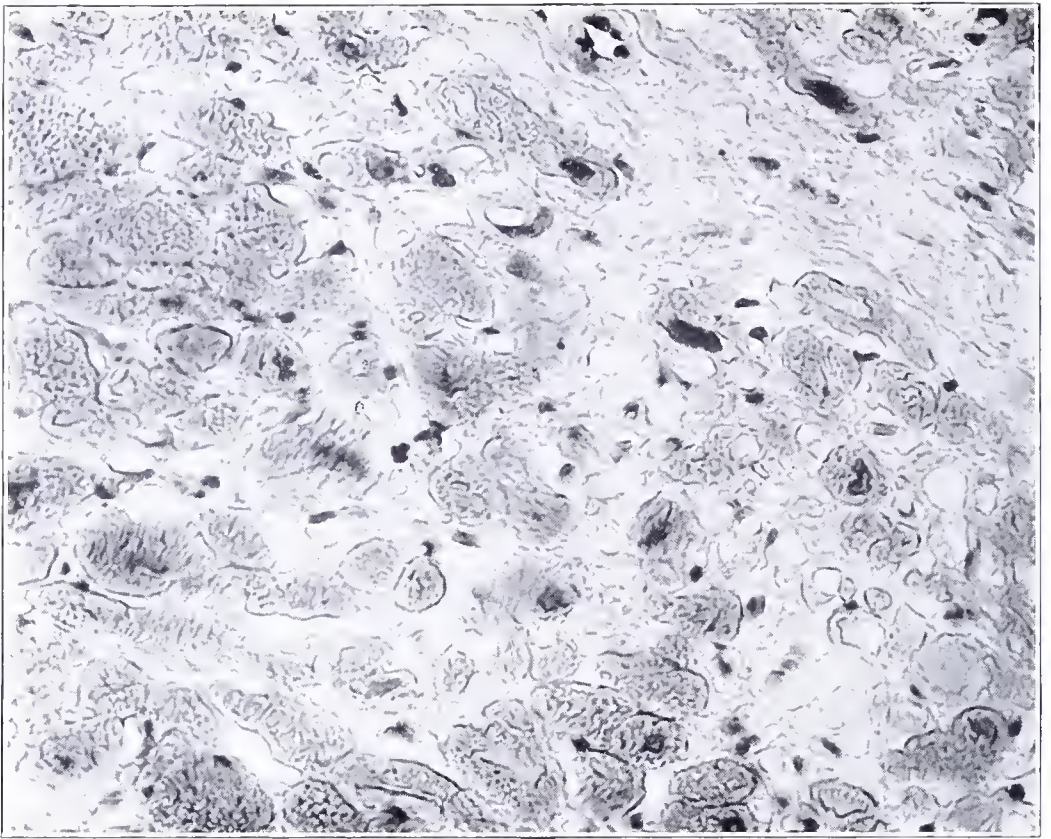


Fig. 7.—Another area in myocardium of case 3, showing marked changes in both heart muscle and bundle of His, more marked in the latter.

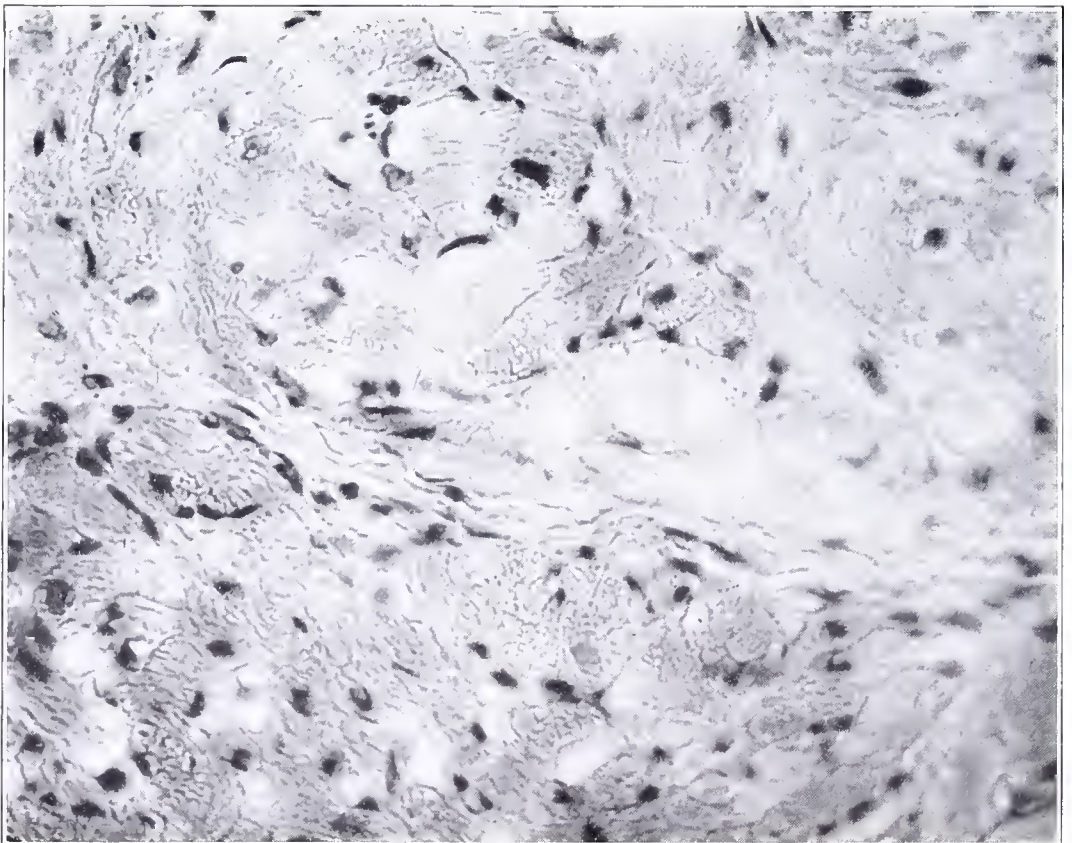


Fig. 8.—Higher power detail of fig. 7, showing marked hyaline change in the fibers of His bundle, in upper part of field.

aspiration of a foreign body; and no preexisting infection. Roentgen-ray examination appeared to indicate the presence of a foreign body. Tracheotomy was performed; no foreign body was seen. There was a mass occluding the trachea just above the bifurcation, thought to be an enlarged thymus. After operation the temperature ran to 100-103.5 F.; the pulse rate, 110-130, irregular. Respiration was 40-48. There were attacks of coughing with thick yellow sputum. The patient had convulsions, and died of cardiac failure on April 23, 1917.

Heart.—The heart was approximately normal in size; the left side was in rigor; the right side was dilated and filled with soft cruor. Other findings were: heart muscle yellowish, especially beneath endocardium; cloudy areas, soft, valves and vessels negative.

Microscopic Examination.—There was a diffuse hyaline change, most marked in the left ventricle and septum; the fibers were homogeneous, and stained poorly; the striation was lost, and the nuclei were slightly pyknotic. A few fibers showed cloudy swellings. No fatty change, no inflammatory reaction, and no evidence of repair or regeneration were present.

Other Organs.—Findings in other organs were: Diphtheric tonsillitis, pharyngitis and laryngitis; streptococcus infection of tracheotomy wounds; acute purulent bronchopneumonia; acute passive congestion and parenchymatous degeneration of all organs; also a thymicolymphatic constitution.

CASE 7.—A boy, aged 3, who had been operated on for mastoid infection, had laryngeal diphtheria. He was given 10,000 units, without effect. Tracheotomy was performed. He died from cardiac syncope during the night.

Heart.—The right side was dilated; the heart muscle was yellowish brown, soft, and translucent.

Microscopic Examination.—There was diffuse hyaline change. The muscle fibers were homogeneous with loss of striations and light staining with eosin. Occasional fibers showed fat droplets. There was no inflammatory reaction, and no evidence of repair or regenerations. The blood vessels were congested.

Other Organs.—There were interstitial emphysema of the mediastinal tissues extending from the tracheotomy wound, diphtheria of larynx and trachea, phlegmonous infiltration of the thyroid and cervical region, acute catarrhal bronchitis, chronic mastoiditis with operation, congenital syphilis (white pneumonia, miliary gummas), lymphoid hyperplasia, atrophy of the thymus, cloudy swelling of the liver and kidneys and general passive congestion.

CASE 8.—A woman, aged 18, had sore throat on Feb. 11, 1918. She had mild delirium, and was toxic. She coughed up a membrane on March 1, 1918. Diphtheria bacilli were present. She received 20,000 units intravenously, and 10,000 subpectorally. Her temperature was 102.3 F. A laryngeal extension of the disease developed, and she received 10,000 units intravenously on March 2. On the 3d there was partial paralysis of the right arm and leg. The reflexes were lost on the right side; 10,000 units more were given. March 4, the temperature was 104.6 F. Cyanosis was marked, respiration was difficult and rapid; the pulse rate was 200 and was irregular. There was a total loss of reflexes and knee jerks. The patient died in cardiac syncope on March 4, 1918.

Heart.—The heart was about normal in size. The muscle was a dark reddish brown, slightly more friable than normal, and cloudy. No fatty change was apparent. The valves and vessels were negative.

Microscopic Examination.—Diffuse hyaline change and cloudy swelling were present. There was no complete necrosis. There were slight fatty degenerative change beneath the endocardium, congestion of the vessels, localized areas of fibrosis, a healed myocarditis, and muscle regeneration.

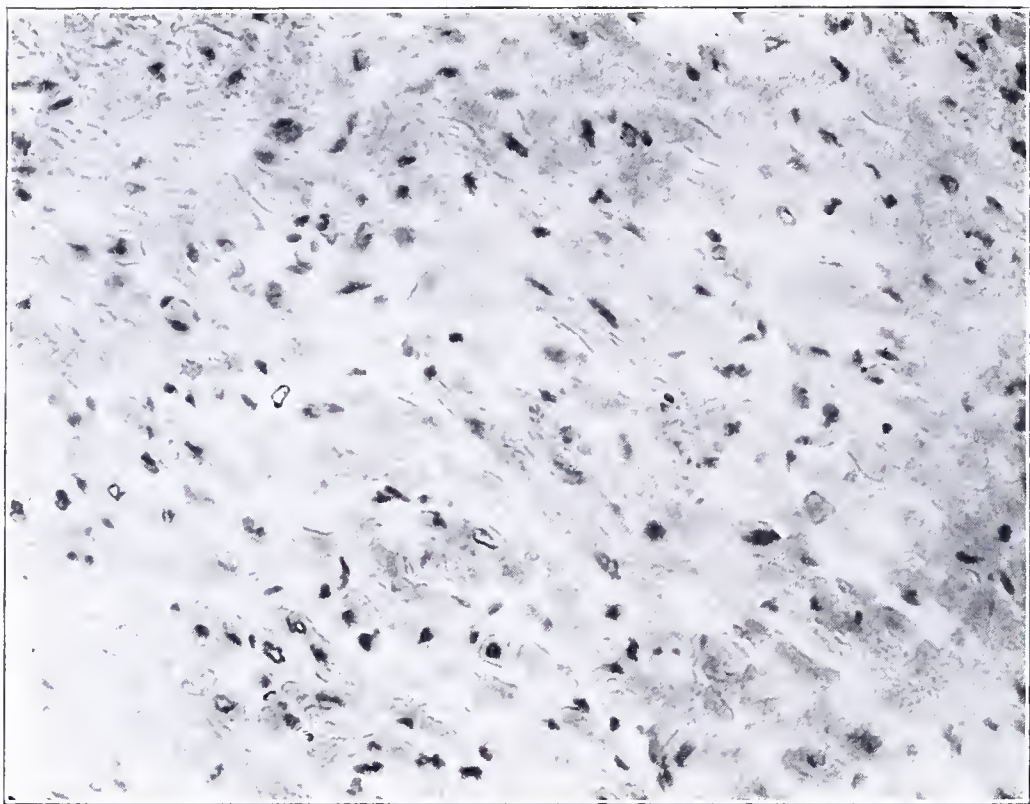


Fig. 9.—Myocardium from case 7, two to three days' duration. Area showing moderate hyaline change; no inflammatory reaction.



Fig. 10.—Area in myocardium of case 7, showing more marked hyaline change in the muscle, loss of striation, homogenization and nuclear changes.

Other Organs.—Findings in other organs were: diphtheria of the tonsils, uvula, tongue, epiglottis and pharynx; thrombosis of the right jugular vein with multiple pulmonary emboli; acute purulent bronchopneumonia; general passive congestion; cloudy swelling of the kidneys.

CASE 9.—A boy, aged 6, on Dec. 11, 1919, showed positive cultures of diphtheria bacilli. He had had "tonsillitis" 4 days before. There was a bloody discharge from the nose. The temperature was septic. Six thousand units were given, followed by 10,000 on the day following. Involuntary micturition occurred. The temperature was 99-100 F.; the pulse rate, 120-130, and irregular; respirations, 30-60. The patient was toxic; he had hematuria, and constant vomiting. He had paralysis of bladder, and delirium. There was marked irregularity of the pulse. The patient died in cardiac syncope on Dec. 26, 1919.

Heart.—The heart was slightly enlarged. Slight fatty degenerative infiltration was present beneath the endocardium. The valve and vessels were negative.

Microscopic Examination.—There was marked diffuse hyaline change of the muscle fibers, more severe in the left ventricle than in the right. The papillary muscles showed marked fatty degenerative infiltration. The vessels were congested. There were no inflammatory reaction, no evidences of repair or regeneration, and no complete necrosis.

Other Organs.—Findings in other organs were: diphtheria of the tongue, pharynx and esophagus; purulent cystitis, ureteritis and pyelonephritis; multiple abscesses of the kidneys; purulent bronchopneumonia; passive congestion and cloudy swelling of all organs; monorchidism.

CASE 10.—A man, aged 22, whose right kidney had been removed 6 months previously, developed erysipelas. On Jan. 7, 1920, a patchy membrane developed in the right tonsil; on the 9th, it had spread over the pharynx, with well-defined cervical cellulitis. Diphtheria bacilli were present; 20,000 units were given intravenously and 20,000 intramuscularly. The patient was toxic; he had a temperature of 103 F.; the pulse rate, 130-201, and irregular; respirations, 30-40. There were casts and albumin in the urine. The patient died of cardiac failure on Jan. 11, 1920.

Heart.—The heart was slightly enlarged; the walls were thin, atrophied. The muscle was yellow-brown, cloudy, and soft. The valves and vessels were negative. Early lipoidosis of the intima of the aorta was present.

Microscopic Examination.—Marked hyaline change and early necrosis were present. No cloudy swelling; no fatty change; no inflammatory reaction; no evidence of regeneration or repair were present. The vessels were congested.

Other Organs.—Findings in other organs were diphtheria of the pharynx; chronic tuberculosis of the bladder, prostate, seminal vesicles and bronchial nodes; miliary tubercles in the lungs, spleen, liver, kidney, tonsils, prostate, peritoneum and pancreas; atrophic arthritis; erysipelas; cervical cellulitis; general passive congestion and cloudy swelling of all organs.

CASE 11.—A boy, aged 13, with a history of congenital syphilis, had had diphtheria 3 weeks before coming to the hospital. He complained of pain in the right side, shortness of breath and cough. There was no membrane in the throat. The pulse rate was 90. His condition became rapidly worse, and there was increasing cyanosis, blood-streaked sputum, Cheyne-Stokes respiration, a pulse rate 130-150, and a temperature of 99-101 F. The pulse at times was regular; at other times, irregular; the apex was outside of the nipple line. There were no murmurs or thrills. The patient died March 24, 1921. A clinical diagnosis of bilateral pneumonia was made.

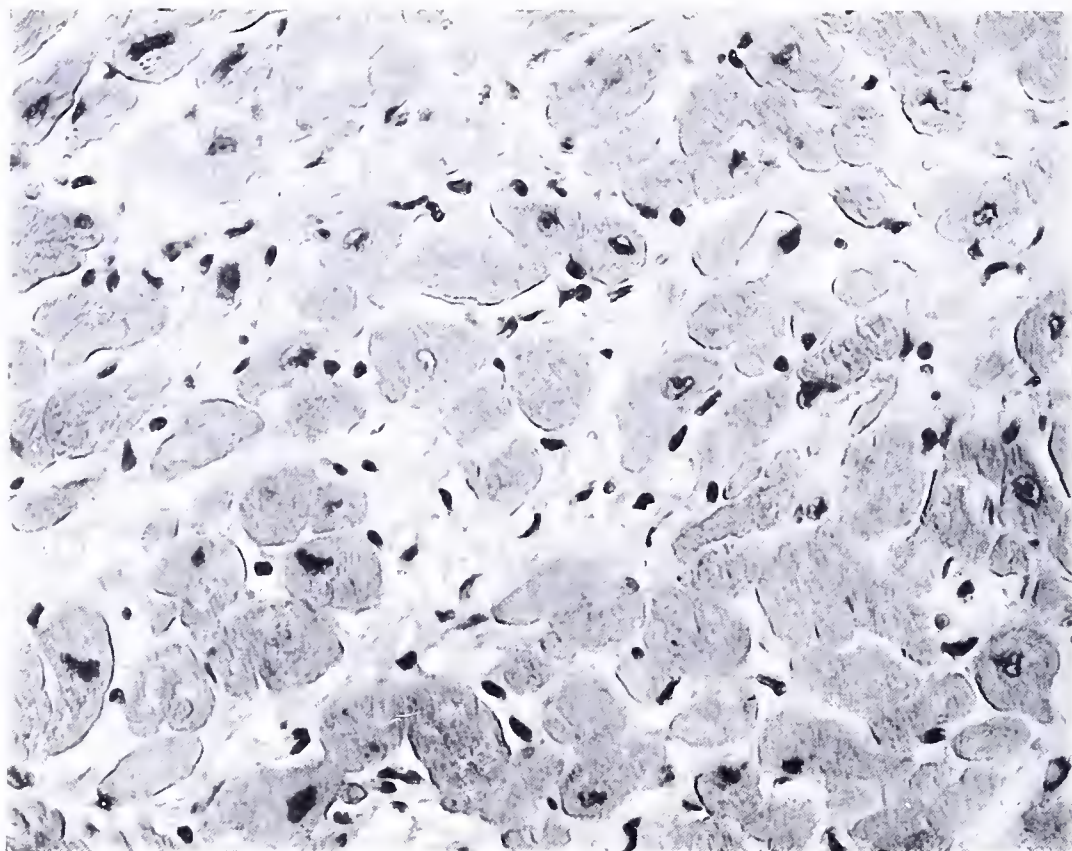


Fig. 11.—Myocardium from case 11. Marked hyaline necrosis and vacuolation of fibers; perimysial nuclei enlarged and stained deeply.



Fig. 12.—Myocardium from case 11. Longitudinal section of heart muscle, showing marked hyaline necrosis, loss of muscle substance, and beginning proliferation of perimysial cells.

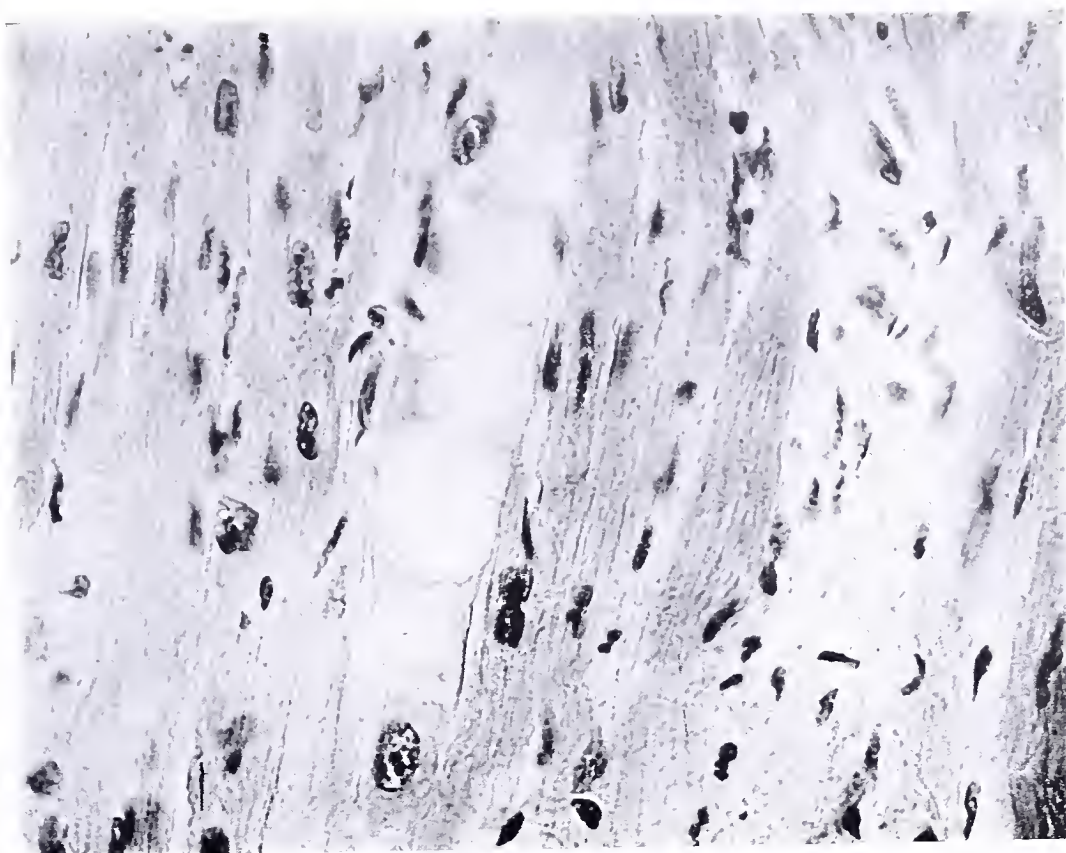


Fig. 13.—Higher power view of myocardium from case 11. Marked hyaline necrosis of portions of the fibers. One fiber broken up into smaller hyaline rounded segments. Increase of perimysial nuclei.

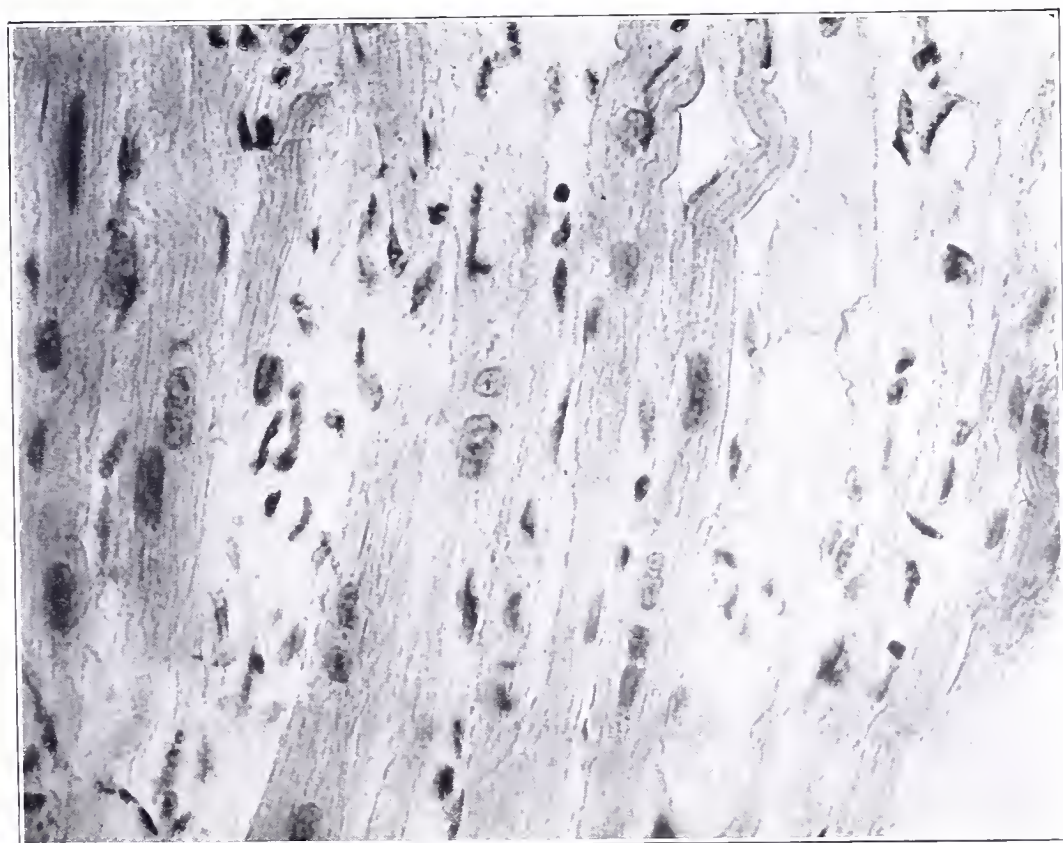


Fig. 14.—Another area in same heart, shewing various stages of hyaline necrosis of the fibers.

Heart.—The heart was greatly enlarged and dilated. It weighed 410 gm. and measured 12.5 x 12.5 x 5.5 cm. The apex was slightly bifid. All cavities and valvular orifices were dilated. The ventricular walls were thin. There was a polypoid parietal thrombus in the left ventricle near the apex. The muscle was yellowish brown, particularly toward the apex, and soft.

Microscopic Examination.—There was a diffuse hyaline change with patches of waxy necrosis in the wall of the left ventricle. Near the endocardium many fibers showed marked fatty degenerative infiltration. There was a parietal thrombus over this area. Throughout the myocardium, individual fibers showed

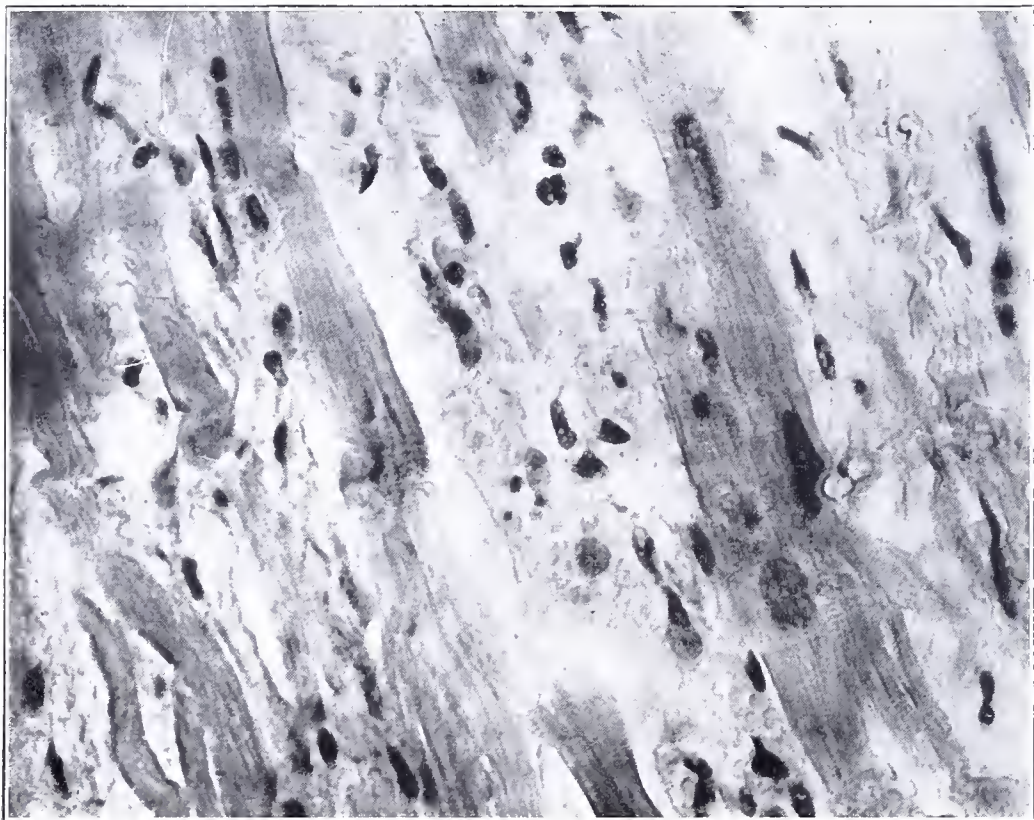


Fig. 15.—Another area in myocardium of case 11, showing higher power details of the degeneration and necrosis of the muscle. Perimysial tubes containing detritus and formative cells.

advanced Zenker's necrosis and marked vacuolation. Partly disintegrated fibers appeared here and there; about these there was edema of the interstitial substance, lymphocyte infiltration and early fibroblastic proliferation. Other findings were: early myocarditis and repair; vessels congested; interstitial tissues edematous; in the areas showing necrotic portions of fibers, the living portions showed large hypertrophic and hyperchromatic nuclei; numerous myoblasts; early muscle regeneration; sections of bundle of His showed marked vacuolation of the fibers, congestion and edema.

Other Organs.—Findings in other organs were: hyperplastic tonsillitis with diphtheric patches in crypts; multiple anemic infarctions of kidneys and

hemorrhagic infarctions of lungs; bronchopneumonia; marked passive congestion of all organs; thymicolymphatic constitution; duplication of the left ureter; ascites. There was no histologic evidence of syphilis.

CASE 12.—A boy, aged 14, two weeks before admission to the hospital had had sore throat and slight fever diagnosed as tonsillitis. Two days before admission he became worse. His throat was sore. He had great difficulty in speaking, and there was regurgitation of food through the nose. There was an extensive dirty gray membrane on both tonsils and the soft palate. The heart sounds were rapid, otherwise good. Ten thousand units were given intravenously, 40,000 intramuscularly, with a slight reaction. The temperature was 101-102 F., the pulse rate, 100-110; respirations, 30. The patient became increasingly toxic and delirious, and was intubated. On the next day he was comatose. His temperature was 105 F., the pulse rate, 160. He died in cardiac syncope on June 22, 1921.

Heart.—The heart was not enlarged. There was rigor of the left ventricle. The right side was dilated. Numerous minute petechial hemorrhages were present beneath the epicardium. The muscle was pale on section. The valves and vessels were negative.

Microscopic Examination.—Early hyaline change and cloudy swelling; slight fatty degenerative infiltration and congestion of the blood vessels were present. No inflammatory reaction and no repair or regeneration were seen.

Other Organs.—Findings in the other organs were: diphtheria of the tonsils, larynx, pharynx, trachea and large bronchi; acute bronchopneumonia; multiple subserous hemorrhages; asphyxia; passive congestion and parenchymatous degeneration of all organs; thymicolymphatic constitution.

CASE 13.—A boy, aged 4, had diphtheria following removal of peanut causing infection. On Nov. 23, 1921, tracheotomy was performed, with removal of peanut from bifurcation. The child was extremely cyanotic, and was revived with difficulty. His condition improved after operation. Five days later respiratory difficulty suddenly increased. Large pieces of membrane were removed from trachea and bronchi. Bacteriologic examination showed pure culture of *B. diphtheria*. Direct smear from membranes showed countless numbers of bacilli. Twenty-five thousand units were given intravenously, and 15,000 more intramuscularly. The child quickly became toxic and cyanotic. Twenty thousand units more given intravenously and 20,000 more intramuscularly. A bronchial cast was passed. A pure culture of bacilli was obtained. The pulse rate was 120-140, very irregular. The temperature was 100-102 F. and respirations 35-40. He died in cardiac syncope Nov. 28, 1922.

Heart.—There was dilatation of all cavities. The muscle was yellowish brown, translucent, soft and tore easily. The patient had a marked tiger heart. The valves and vessels were negative.

Microscopic Examination.—There were marked fatty degenerative infiltration and hyaline change. The fatty change was most marked in the left ventricle near the endocardium. There was marked tiger heart, and marked congestion of the vessels. No inflammatory reaction, and no repair was seen.

Other Organs.—Findings in other organs were: aspiration pneumonia in the right lung; tracheal and bronchial diphtheria; interstitial emphysema of the left lung; diffuse inflammation of peritracheal and mediastinal tissues; lymphatic constitution; passive congestion and parenchymatous degeneration of all organs; Meckel's diverticulum.

CASE 14.—A girl, aged 7, entered the hospital with a diagnosis of diphtheria. She had been ill for 2 days, and was toxic. The cervical glands were enlarged. There was a membrane in the pharynx. She was given 10,000 intravenously, and

40,000 in the buttocks. Immediately she had a severe chill, strident respiration, stiff extremities, and involuntary expulsion of feces. She became restless and cyanotic, and died within 15 minutes. Injection of epinephrin was of no avail.

Heart.—The heart was moderately dilated. There was rigor mortis contraction. The muscle apparently was normal.

Microscopic Examination.—All of the muscle fibers showed a more or less marked hyaline appearance, with pyknosis and fragmentation of many of the nuclei, although the chromatin was not completely lost anywhere. There was early Zenker's necrosis. The striations were lost. There were a few minute heart droplets.

Other Organs.—Findings in the other organs were: tonsillar diphtheria; hyaline necrosis of the striped muscles of the pharynx and larynx; thymicolymphatic constitution; parenchymatous hypertrophy of the thyroid; marked congestion of the lungs with multiple hemorrhages; acute catarrhal laryngitis, tracheitis, and bronchitis; old tubercles in lungs and bronchial nodes; oxyurids in the appendix.

CASE 15.—A boy, aged 2½, had laryngeal papilloma removed with tracheotomy. A diagnosis of diphtheria was made on Dec. 15, 1922. Twenty thousand units were given intravenously and 20,000 intramuscularly. One hour later the temperature rose rapidly to 107° F. He developed marked dyspnea, and had epileptiform convulsions; in the second hour, pulmonary edema and convulsions became more severe. Six hours after the injection the child stiffened and died. Epinephrin had no effect. The heart was not examined during this period; at entrance it was negative.

Heart.—The heart was dilated. There were small petechial hemorrhages beneath the endocardium, yellowish brown in color. No fatty change was visible to the naked eye. The valves and vessels were negative.

Microscopic Examination.—Examination revealed: diffuse early hyaline change; subepicardial fatty change in the right ventricle; congestion of vessels. No inflammatory reaction, and no repair were seen.

Other Organs.—Findings in the other organs were: diphtheria of the tonsils and trachea; thymicolymphatic constitution; miliary tubercles in the bronchial and mesenteric nodes; older tuberculous focus in the base of the right lung; recurrent laryngeal papilloma with tracheotomy; localized hypertrophy of the thyroid; marked congestion, edema and multiple hemorrhages in the lungs; passive congestion and parenchymatous degeneration of all organs.

CASE 16 (Dr. Hektoen).—A boy, aged 2, died 2 days after admission to the hospital. Three days before, he had been taken ill with sore throat and vomiting. No physician was called until the 3d day, when 20,000 units were given, and the child was taken to the hospital, where 20,000 units more were given. The tonsils were large and covered with a membrane which extended over the uvula and posterior pharyngeal wall. On the day after admission, the pulse became slow, weak and irregular, and remained irregular in spite of hypodermic injections of caffein and application of heat externally. Heart beats were skipped, and there were premature contractions. Death occurred on the 6th day of illness. Necropsy showed changes only in the throat, heart and lymphoid tissue of the intestinal tract, as follows: diphtheria of throat, with some edema of the epiglottis; pale, flabby and friable myocardium. Weight of heart, 70 gm. There was hyperplasia of the lymphoid tissues of the intestinal tract. No pneumonia.

Microscopic Examination.—Sections of the myocardium showed an intense diffuse hyaline change. The muscle fibers were swollen, hyaline, homogeneous, with loss of striation and staining-power. The nuclei were deformed and

pyknotic and in the most hyaline portions of the fibers completely lost. Occasional fibers showed cloudy swelling, staining heavily with eosin. There were a few fat droplets. In the middle layer of the left ventricle, the parenchymatous changes were most marked. A portion of the fibers showed complete hyaline necrosis. The interstitial tissue about such fibers was edematous and showed early lymphocyte infiltration and fibroblastic proliferation (early myocarditis). There was no regeneration of the cardiac muscle. The portions of the bundle of His included in the blocks taken showed the same changes as in the other portions of the myocardium.

SUMMARY OF CASES

The ages of the patients were 25, 40, 3, 2, 31, 2½, 3, 18, 6, 22, 13, 14, 4, 7, 2½ and 2 years. The duration of the infection varied approximately as follows: 1-2, 2-3, 4, 6, 12, 14, 15, and 16 days, and 2½, 3 and 6 weeks.

It is of interest that two of the cases developed after the removal of an aspirated peanut. They were both characterized by the severity of the diphtheric tracheitis and bronchitis, which extended into the smaller bronchioles, and by the virulency of the organisms, as shown by the enormous numbers of diphtheria bacilli in the membranes. In both cases, the nature of the bacilli present was demonstrated bacteriologically. In a third case, the child had had a previous tracheotomy for recurrent papilloma of the larynx. It would appear from these cases that the mechanical trauma caused by such operations favors the development of diphtheria either from bacilli carried by the patient or received after the operation. In a fourth case, with supposed foreign body obstruction, no foreign body could be found on examination or at necropsy; and it is most probable that this case was one of diphtheria from the beginning.

Two of the patients died following the administration of antitoxin. Both children showed well-marked features of the thymicolymphatic constitution with areas of parenchymatous hypertrophy in the thyroid resembling that of exophthalmic goiter. The pathologic picture of anaphylactic death was not shown in these cases. No characteristic increase in the distribution of leukocytes in the hepatic capillaries was present. We regard the death in these cases as the result of the combination of diphtheria and the thymicolymphatic heart, the antitoxin injections serving as any other exciting factor (burns, cold, emotion, etc.) to bring about the cardiac form of death.

All of the 16 patients died from cardiac failure. Disturbances in rate, rhythm and heart sounds were present in all, with a general picture of dilatation. No especial disturbances of conduction were noted in any

one. The emergency character of many of the cases prevented any especial cardiac study clinically, and none was attempted. Nevertheless the clinical impression received from each case was that it was one of "cardiac failure." The necropsy findings bear this out. In every case, there was more or less marked cardiac dilatation, involving all of the heart chambers, or especially marked on the right side.

No endocarditis was seen in any one of the cases.

In one case only was there a mural thrombus. This was situated in the left ventricle near the apex, over an area of severely damaged myocardium, the degeneration and necrosis extending to the endocardium. In one case, there was a thrombosis of the right jugular vein with multiple pulmonary embolism.

It is a striking fact that 9 of the patients presented well-marked features of the thymicolymphatic constitution, again an emphasis of the liability of this constitution to death from acute infections.

In nine of the cases there was a more or less pronounced bronchopneumonia. In the rapidly developing cases with early death, pneumonia was absent or agonal; in the more severe and prolonged cases and in those with aspiration of foreign material, it was more marked. The cardiac embarrassment was no doubt increased in the latter cases, but it is important to note that the cardiac symptoms may be as pronounced in the cases without pneumonia as in those with it.

The other changes consisted of a generalized passive congestion, often with petechial hemorrhages, especially marked in the lungs, parenchymatous degeneration of liver and kidneys (cloudy swelling or degenerative fatty infiltration), and lymphocyte exhaustion of germ centers of spleen and lymphnodes. In those cases in which they were examined, the striped muscle fibers of the pharyngeal and laryngeal muscles showed a marked Zenker's necrosis.

The essential pathologic lesion, aside from the local diphtheria process, in every one of the cases was a marked parenchymatous degeneration of the heart muscle, varying in degree with the severity of the process and with the stage of duration.

In every case hyaline degeneration or necrosis was present. This is the one constant parenchymatous lesion. In the early deaths, this may be the only histologic lesion in the myocardium. It is characterized by more or less tumefaction with loss of striation and staining power (eosin) and increased refraction of portions or all of the fibers. The nuclei may show little change in the early cases, but are usually con-

tracted, deformed and more or less pyknotic. In the more severe or prolonged cases the picture of a hyaline, waxy or typical Zenker's necrosis is presented. The fibers or affected portions of such are swollen, waxy, yellowish or clear, distorted, fragmented, containing large irregular vacuoles, or in longitudinal section often partly frayed out, or divided into roundish hyaline segments, with complete loss of nuclei in the affected portions. Living portions of muscle alternate with necrotic portions so that apparent gaps or defects occur in the fibers. Many of the hyaline dead muscle portions have a high refractive index exceeding that of amyloid. The dead portions of the muscle act as a foreign substance stimulating phagocytosis by leukocytes and the production of foreign-body giant cells in the stroma. The perimysial tubes become filled with detritus and cells, at first chiefly polymorphonuclear leukocytes, later by a variety of forms, fibroblastic and myoblastic. The hyaline degeneration may vary from slight, barely recognizable changes to the picture of complete necrosis. With van Gieson's stain, the fibers showing certain stages of waxy necrosis may stain reddish with the acid fuchsin, while losing their affinity for eosin. The more yellowish the muscle appears in the hematoxylin and eosin stains, the redder it will stain with van Gieson's stain.

The hyaline change may be the only parenchymatous lesion present, or it may be associated with fatty degenerative infiltration, or with cloudy swelling, or with both of these changes. Marked fatty change was found in only two of our cases. It occurs especially toward the endocardium of the left ventricle and often involves the papillary muscles especially, giving the picture of a tiger heart microscopically, which is often not recognized in the gross because of the associated hyaline change. In our cases, fatty degenerative infiltration (so-called fatty degeneration) is certainly not the essential or most frequent lesion. Its association with hyaline degeneration may be purely secondary to associated nutritive conditions (anemia, asphyxia, etc.).

Likewise, cloudy swelling is only an associated change, much less frequent than fatty change. In one case only of our series did it occur extensively in the myocardium, and its association is probably due to nutritional factors.

Simple necrosis of individual fibers also occasionally occurs. Vacuolation not due to fat, apparently hydropic degeneration, also occurs in association with marked interstitial edema. Local liquefaction of the dead portions of the fibers is also occasionally seen. It appears to follow

waxy necrosis. No widespread liquefaction resembling Eppinger's myolysis was ever observed. From the histologic study of these 16 cases, I conclude that parenchymatous injury in the form of hyaline change of the heart muscle is the most common essential lesion produced by diphtheria toxin in the heart muscle. The same thing is probably true of the striped muscles affected by it (diphtheric paralysis).

Congestion of the coronary vessels and capillaries, small hemorrhages and edema of the interstitial tissues are associated with the parenchymatous lesion.

In the delayed cases, but even as early as the 3rd to 6th day, lymphocytes and polymorphonuclears and occasional eosinophiles may collect about the small vessels in the stroma of the damaged portions of muscle. No marked collection of eosinophiles was seen in our series. As the interstitial edema increases, the picture of Zenker's necrosis with vacuolation, or of simple hydropic degeneration alone, appears. The muscle fibers become fragmented, or frayed, partly liquefied and absorbed. Plasma cells and fibroblasts appear in the stroma; the picture of a well-developed myocarditis results. This is apparently wholly reparative in nature. Such reparative changes may be well marked at the end of the 2d week, as in case 2; on the other hand they may be much delayed, as in case 3, at the end of 6 weeks.

Muscle Regeneration.—Evidences of muscle regeneration were seen in 3 of the cases, of 2, 3 and 3 weeks' duration, respectively. In the case of longest duration, 6 weeks, repair and regeneration were apparently delayed, or there may have been a progressive injury without repair. This patient showed the severest type of diphtheric paralysis of the striped muscles, and no evidences of repair were found in the latter.

Near the necrotic or degenerated portions of the heart muscle, the nuclei of the muscle show a great variety in size and form. They increase in length and show many evidences of longitudinal splitting in every possible stage of such a division. The living muscle substance bordering on the injured area also undergoes a longitudinal splitting into muscle bands containing nuclei; these bands grow into the perimysial tubes, filling these up, replacing the cell detritus, and connecting with the living muscle on the other side of the defect. Muscle bands without nuclei in their substance but accompanied by myoplastic nuclei also extend into the tubes occupied by the dead muscle substance. These bands lie at the periphery of the tube, and in some cases appear to form

a hollow cylinder enclosing the remains of the dead muscle substance. Transition forms from tubes with a single peripheral syncytial layer to those filled with a number of longitudinal bands occur. The myoplastic nuclei are at first large, oval or spindle-shaped, staining less deeply than mature heart-muscle nuclei, but later become contracted and more deeply staining. All possible transition stages between these and mature nuclei occur. The young protoplasmic bands at first show no evidences of striation, but in the more mature forms the striations appear as fine cross-striations near the poles of the nuclei. Bulbous swellings filled with myoplastic nuclei occur occasionally at the living ends of the muscle defects, and these can also be found showing longitudinal splitting. We have seen all of the appearances illustrated by Heller⁷⁷ in his article on the regeneration of the heart muscle. The defects in the muscle caused by hyaline necrosis may ultimately be bridged by a number of new muscle bands apparently uniting with the living muscle on the other side of the defect. The sharp delimitation of the muscle in perimysial or sarcolemmar tubes was shown in our cases as in those of Anitschow and Heller. These appearances, as Heller has pointed out, seem contradictory to the present accepted view that the heart muscle has no true sarcolemma. There is a close similarity between the process of regeneration of the heart muscle and that of the peripheral nerve trunks.

With the regeneration of the heart muscle there takes place a parallel proliferation of the stroma. To what extent the newly-formed connective tissue persists in the form of localized fibrosis, or whether the proliferation of the stroma is necessary only as a congenial environment for the development of the new muscle fibers bridging the defects, cannot be decided here. In the less severely damaged hearts with a minimum of muscle repair, it is possible that complete regeneration without fibrosis may occur. In the more severe cases with extensive muscle necrosis, such as is likely to be associated with any severe post-diphtheric paralysis of the voluntary muscles, the possibility of cardiac fibrosis and impairment of cardiac function later in life must be borne in mind. This is shown by the clinical evidence already at hand. Nevertheless, some persons who have had severe diphtheria in childhood with marked cardiac weakness do come to the age of 60 or more without clinical symptoms of cardiac weakness. Some patients with considerable myocardial injury due to diphtheria recover so completely that the demands of an active life on the heart are fully met. The clinical evidence favors complete regeneration or compensating hypertrophy in

some cases. In any case, the final outcome will depend on a number of factors, not only the degree of the lesion, but its extent and location, and the completeness of the regenerative and reparative processes.

The bundle of His was especially studied in two cases, and the sections from all of the cases have been gone over again with reference to the conducting bundle. In three cases the bundle showed the same hyaline changes as in the general myocardium, but with a more marked interstitial edema, and vacuolation of the fibers. In the other cases, hyaline degeneration of the same degree as that in the myocardium was seen in the bundle. No evidence was seen arguing for any especial affinity of the toxin for the conducting apparatus.

CONCLUSIONS

The essential lesion of the heart in diphtheria is a toxic parenchymatous hyaline degeneration or necrosis, associated frequently with fatty degenerative infiltration and less frequently with cloudy swelling or a simple necrosis. The latter lesions are most probably due to accompanying nutritional conditions. Following the lesion, there is (if the patient survives) a reparative inflammatory process (myocarditis) accompanied by muscle regeneration. Either a complete regeneration or a fibrosis may result. The toxin of diphtheria may damage the conducting mechanism as well as the contractile. No especial affinity is shown pathologically for either apparatus.

The conflicting pathologic descriptions given in the literature can be unified, and the diphtheria heart given a definite entity of a primary toxic parenchymatous degeneration most frequently of a hyaline nature followed by a reparative inflammation (myocarditis) with muscle regeneration. The histologic picture in any given case will depend on the duration and stage of the infection, upon the degree of toxic injury to the muscle, the associated nutritional conditions, and the degree of muscle regeneration and accompanying fibrosis.

MICROCOCCUS OVALIS

LXX. STUDIES IN BACTERIAL METABOLISM

ARTHUR ISAAC KENDALL AND REBA CORDELIA HANER

From the Department of Bacteriology and Patten Research Foundation, Northwestern University Medical School, Chicago, Ill.

Micrococcus ovalis, an organism commonly found in the intestinal flora of normal nurslings and exhibiting a characteristic morphology, was isolated, described and named by Escherich.¹ Several years later it was rediscovered by Thiercelin² and referred to as *Enterococcus*. There seems to be little doubt that *Micrococcus ovalis* and the *Enterococcus* are identical.³

The morphology of the enterococcus is quite distinctive and reminiscent of the pneumococcus. It is characteristically an elongated coccus, occurring typically in pairs, with the apposed ends slightly flattened and the opposite ends somewhat pointed. This feature is frequently lost, or materially modified, on prolonged cultivation outside the human body.

Micrococcus ovalis stains readily with ordinary anilin dyes, and retains the Gram stain. Usually the pairs of cocci, colored by any method, exhibit a faint, but clearly discernible unstainable line separating the two elements.

Capsules are usually not demonstrable, even if the organisms are cultivated in albuminous mediums; indeed, with the exception of Lewkowicz,⁴ who claims to have stained capsular substance, presumably in a freshly isolated strain, capsules do not seem to have been demonstrated by any investigator.

The similarity of morphology, both with respect to size and shape, between the enterococcus and the pneumococcus doubtless has led to some of the reported findings of pneumococci in intestinal contents and feces. Pneumococci must occur occasionally in the intestinal con-

Received for publication, April 1, 1924.

¹ *Darmbakterien des Säuglings*, 1886, p. 89.

² *Compt. rend. Soc. de biol.*, 1899, 55, 269.

³ Tissier: *Recherches sur la flore intestinale des nourrissons*, 1900.

⁴ *Centralbl. f. Bakteriol.*, 1901, 29, p. 635.

tents, being swallowed and passed downward during the digestive process, and there is little reason to believe they would be dissolved by the low concentration of bile normally found at any level of the intestinal tract. The enterococcus, on the other hand, is resistant to solution by bile. A further point of resemblance between the two is the shiny, gray, and relatively luxuriant growth on Loeffler's blood serum slants exhibited by both organisms. In fluid mediums, also, especially those containing carbohydrates, both coccal forms grow and



Fig. 1.—*Micrococcus ovalis*, $\times 1,200$. Lactose broth culture, 48 hours.

appear habitually as short, streptococcoid chains. The diplococcoid arrangement, however, is usually clearly discernible.

Figures 1-3, all magnified 1,200 times, illustrate the morphology of *Micrococcus ovalis* and the pneumococcus with great definiteness. *Micrococcus ovalis* (figs. 1 and 3) is somewhat larger than the pneumococcus and distinctly less pointed at the ends, although both of these distinctions are quantitative rather than qualitative. The streptococcoid arrangement of *Micrococcus ovalis* in fluid mediums is well shown in fig. 1. The pneumococcus also forms short chains under similar conditions.

Immunologically, pneumococci (types 1-3 inclusive) do not react specifically with ovalis serums, and ovalis types thus far encountered do not react with any of the serums specific for the pneumococcus types.⁵

The pathogenesis of *Micrococcus ovalis* is as yet a matter of controversy. A few cases of diarrhea have been reported,⁶ which show on careful analysis a distinct overgrowth of this microbe associated with the condition. This fails to be convincing proof of a primary etiologic relationship to the morbid process, however. Any normal parasitic organism living habitually on a mucous membrane in free communication with the outside of the body may invade underlying tissues, if some antecedent or accessory agent opens a pathway through epithelia which normally resist their passage.⁷

Notwithstanding the considerable literature which has grown up around *Micrococcus ovalis*, comparatively little is known about the microbe. This is probably attributable, in part at least, to its rather distinctive morphology, which renders a presumptive recognition fairly simple.

Nevertheless, considerable importance attaches to the enterococcus, not only because it is a normal inhabitant of the infantile canal, but also because it may be detected in the duodenal contents of adults. Thus, in a series of 73 duodenal specimens, withdrawn through a duodenal tube with proper precautions, *Micrococcus ovalis* was isolated and identified in 58, or nearly 80%. A survey of the clinical features of these cases fails to reveal any tangible relationship between the occurrence of the organism in the duodenal area and gall bladder infection or involvement. On the contrary, the enterococcus appears rather in the light of a normal resident in the duodenum. This does not of course preclude the possibility of its becoming temporarily at least an invader. The frequent occurrence of *Micrococcus ovalis* in the duodenum, as will appear from its chemistry, to be discussed later, is plausibly attributable to the relative richness of the duodenal contents in carbohydrates during digestive periods. At lower levels, where carbohydrates are absent from the intestinal contents for varying intervals, the organism would fail to find its requisite pabulum; it is an obligately fermentative microbe.

⁵ The Cole types are here referred to.

⁶ Kendall: Bacteriology, General, Pathological and Intestinal, 2nd ed., p. 264.

⁷ Kendall: Jour. Infect. Dis., 1923, 32, p. 341.

Of the studies made on *Micrococcus ovalis* to the present time, that of Dible⁸ is the most comprehensive. He found a commonly occurring "type" organism and 3 variants. Among the *ovalis* cultures obtained from duodenal contents, one of us (Haner) obtained not only the Dible type of variants, but also 3 more variants, all of which are given in the table, using the Dible characteristics, except that starch is substituted for dulcitol. The latter alcohol is not fermented by any member of the group thus far encountered.

For purposes of identification, the fermentation of lactose is needless in light of the acid coagulation of milk. Salicin is of some importance. All the *ovalis* strains thus far encountered ferment it, whereas many, if not indeed a great majority of, staphylococci fail to utilize this glucoside for energy. Inulin has some value as a

TABLE 1
FERMENTATION REACTIONS OF *MICROCoccus ovalis* TYPES

	Milk Clot	Man- nitol	Lac- tose	Saccha- rose	Raffi- nose	Inulin	Starch	Salicin	Hemo- lysin
Type organism:	+	+	+	+	—	—	—	+	—
Variant I.....	+	+	+	—	—	—	+	+	—
Variant II.....	+	—	+	+	—	—	—	+	—
Variant III.....	+	—	+	—	—	—	—	+	—
Variant IV.....	+	+	+	+	+	+	—	+	—
Variant V.....	+	—	+	+	+	—	—	+	—
Variant VI.....	+	—	+	+	+	±	+	+	—

classifying agent, although a decided majority of strains fail to utilize this polymer of levulose. The value of raffinose as a diagnostic agent is distinctly impaired through the presence of small amounts of glucose found as an impurity in many samples.⁹

At first sight the fermentation of inulin by variants IV and VI might suggest relationship with the pneumococcus. The insolubility of the organism in bile, the absence of the green halo around colonies on blood agar plates and other characteristics referred to previously make the differentiation between the enterococcus and the pneumococcus a substantial one.

The significance of these variants of the enterococcus and the weight that should be attached to their respective vesatilities with reference to carbohydrate configurations and utilization cannot be stated at this

⁸ Jour. Path. & Bacteriol., 1921, 24, p. 1.

⁹ As small an amount as one part of glucose in 1,000 of culture medium is readily detected. Kendall and Yeshida: Jour. Infect. Dis., 1923, 32, p. 355.

time. The quantitative changes in titratable acidity induced by *Micrococcus ovalis* in mediums having some dietary interest are shown in table 2. It is of interest to note that the acidities are on the whole materially less than those characteristic of *B. bifidus*¹⁰ and *B. acidophilus*.¹¹

The identification of pathogenic bacteria which habitually are tissue invaders,⁷ and their differentiation from parasitic microbes, involves or may involve two distinct procedures,¹² the relative values of which for diagnostic purposes are peculiar for each organism.

In general, the chemical architecture of a microbe is a constant one. Some well-known pathogenic bacteria are so alike in this respect that they produce identical, or nearly identical, serologic reactions in animals. Thus, any typhoid bacillus, injected at proper intervals and in appropri-

TABLE 2
QUANTITATIVE FERMENTATION REACTIONS OF MICROCOCCUS OVALIS

C e N/1 Acid per 100 C e.	Days	Plain Broth	Glucose Broth	Sucrose Broth	Starch Broth	Plain Gelatin	Milk
Control.....	-0.2	-0.2	-0.2	-0.2	-0.1	+1.8
<i>Micrococcus ovalis</i> type organism.....	1	+0.1	+1.8	+1.1	+0.1	+0.3	+4.0
<i>Micrococcus ovalis</i> type organism.....	3	+0.0	+2.5	+1.3	+0.3	+0.2	+5.7
<i>Micrococcus ovalis</i> type organism.....	6	-0.3	+2.7	+1.3	+0.5	+0.5	+5.1
<i>Micrococcus ovalis</i> type organism.....	9	-0.3	+3.1	+1.7	+0.1	+0.6	+5.3

ate ascending amounts, will induce in an experimental animal specific antibodies which will agglutinate, or dissolve, practically all typhoid bacilli and none other in high dilutions. Meningococci and pneumococci, on the other hand, differ somewhat from the typhoid bacillus, so far as available evidence indicates, in that serologic strains may be detected among current strains, each quite distinct from the others. Thus, 3 specific serologic pneumococcus types are recognizable⁵ and at least 4 serologic meningococcus groups are discernible.¹³ As each type induces antibody formation in experimental animals which is specific, in high dilution at least, to eliminate group antibodies, and may be used to identify the particular strain, the serologic recognition

¹⁰ Kendall and Haner: *Ibid.*, 1924, 35, p. 16.

¹¹ Kendall and Haner: *Ibid.*, 1924, 35, p. 28.

¹² Organisms that produce specific soluble toxins are excluded from this discussion.

¹³ Medical Research Committee Report, 1920, 50, p. 128.

of the pneumococcus or meningococcus family requires access to the several specific type serums.

It should be mentioned in passing that the discovery of serologic strains within a pathologic "species" of microbe is a comparatively new development in immunology and deserves to be discussed with some restraint pending more thorough study. Thus, numerous dysentery-like organisms, differing somewhat both in their serologic and cultural reactions, are known, and it is problematical where to draw the line, if indeed a distinction can be made, between frankly pathogenic



Fig. 2.—*Micrococcus ovalis*, $\times 1,200$. Glucose agar slant.

strains and those which may be in a process of evolution toward or away from pathogenesis. Herein lies a most important field for future study.

The meningococcus and pneumococcus types, however, seem to be fairly well established, and in seeking for some hypothesis which shall explain the apparent paradox of 4 distinct serologic meningococcus entities and 3 pneumococcus entities, which are alike morphologically and culturally,¹⁴ but quite unlike in their production of specific anti-

¹⁴ *Pneumococcus mucosus*, type 3 of the pneumococcus group, is an exception to this statement.

bodies, attention must be redirected to the chemical architecture of the several types.

It has long been known that protein injected into experimental animals stimulates antibody formation of high specificity; it would seem to be logical, therefore, to attribute the specificity of the meningococcus and pneumococcus types to respective differences in the protein architecture of the microbes themselves. This suggests that the chemical architecture of current strains of typhoid bacilli is a comparatively

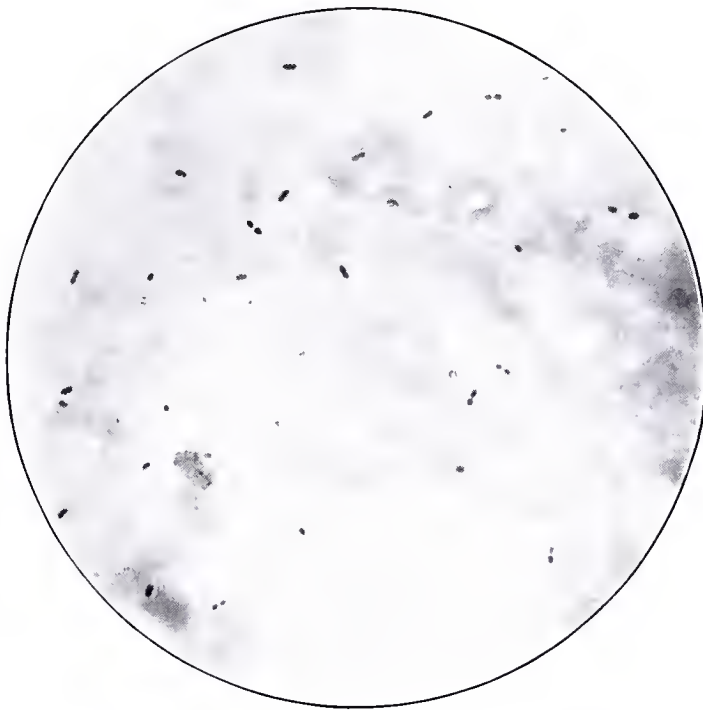


Fig. 3.—Pneumococcus, $\times 1,200$. Heart blood of a mouse.

fixed quality, whereas several protein complexes are discernible among the current strains or types of pneumococci and meningococci.

Viewed from this angle, the identity of a unicellular microbe would seem to depend on the identity—the chemical architecture, in other words—of its protein substance, or to some very unchangeable nitrogenous constituent intimately associated with its protein substance. If such proved to be the case, the change of one microbic “species” into another, even a closely related one, would seem to predicate a profound alteration in the chemical architecture of the microbe.

The versatility of the microbe in respect to its ability to utilize various substances for its energy is an inherent property of this chemical structure. It is not without significance that those bacteria that are progressively pathogenic—that incite diseases communicable from host to host—are rather limited in their choice of structural and energy foods, conforming rather closely to those available in the tissues where they habitually multiply.

Attention is redirected at this point to an earlier communication in which the specificity of action of pathogenic bacteria, so far as available evidence shows, depends on the utilization of protein of the respective microbes for their energy requirements. Most bacteria form the chem-

TABLE 3
NITROGENOUS METABOLISM OF *MICROCOCOCCUS OVALIS*
Type Organism:

Mg. per 100 C c.	Days	Control Plain Broth	Plain Borth	Control Glucose Broth	Glucose Broth	Control Plain Gelatin	Plain Gelatin	Control Milk	Milk
Total nitrogen.....	1	161	161	161	161	721	721	476	476
Amino nitrogen.....		20.3	25.9	20.3	24.5	33.6	34.3	17.5	20.3
Ammonia nitrogen...		2.1	7.7	2.1	6.3	2.8	9.1	4.9	4.9
Total nitrogen.....	3	161	161	161	161	721	721	476	476
Amino nitrogen.....		20.3	25.9	20.3	24.5	33.6	39.9	17.5	23.8
Ammonia nitrogen...		2.1	9.1	2.1	7.7	2.8	9.1	4.9	5.6
Total nitrogen.....	6	161	161	161	161	721	721	476	476
Amino nitrogen.....		20.3	30.1	20.3	24.5	33.6	44.8	17.5	26.6
Ammonia nitrogen...		2.1	9.1	2.1	7.7	2.8	9.8	4.9	5.6
Total nitrogen.....	9	161	161	161	161	721	721	476	476
Amino nitrogen.....		20.3	32.2	20.3	28.0	33.6	45.5	17.5	28.0
Ammonia nitrogen...		2.1	9.8	2.1	7.0	2.8	10.5	4.9	5.6

ical constituents of butter milk (lactic acid) when they utilize carbohydrate for energy.¹⁵ This phenomenon is a function of the protoplasm, not a difference in the specific protein architecture of the organism.

With these characteristics in view, a study of the metabolism of a strain of *Micrococcus ovalis* was made by one of us (Haner) to determine the nature of the nitrogenous interchange of the organisms with the nutritive environment. The customary mediums, milk, plain and carbohydrate broths, were inoculated and studied in accordance with the methods customarily employed for this purpose.¹⁶ The results, in abbreviated form, follow: They are expressed as milligrams of nitrogen per 100 c c. of culture medium.

¹⁵ Kendall: Am. Jour. Med. Sci., 1918, 156, p. 157.

¹⁶ Kendall: Jour. Infect. Dis., 1922, 30, p. 211.

DISCUSSION

The results of this study are quite sharply defined. They confirm previous qualitative observations and throw additional light on existing information. *Micrococcus ovalis* is not a proteolytic organism in the acceptable usage of that term. Its action on nitrogenous substances, as shown by the changes it induces even in plain broth, are so slight as to be scarcely measurable.

It grows but feebly in ordinary nitrogenous, sugar-free mediums. The intimate nature of the changes it induces in such menstrua are unknown, but it may be stated that no amino acids or complexes thereof, are so altered that aromatic decomposition products, such as indol or phenol, are formed. It is, therefore, presumably not a contributor to intestinal putrefaction.

The addition of carbohydrates from which the various strains may derive energy increases the growth of the microbes materially and the products of fermentation, among which lactic acid is prominent, are those characteristic of the alimentary flora of the normal nursling. The organism agrees in essential details with the chemical characteristics of normal intestinal lactic acid bacteria.

It is interesting to note the apparent resistance of the microbes to the powerful digestive enzymes of the upper intestinal tract. Even dead enterococci appear to be unaffected by intestinal enzymes to a marked degree, but this property is apparently shared with normal and pathogenic intestinal bacteria in general. The effect of intestinal juices on alien microbes, as *B. bulgaricus*, remains to be elucidated. In the monkey, *B. bulgaricus* fails to reach the lower levels of the intestinal tract in a viable condition.¹⁷

SUMMARY

The observations recorded above would seem to justify the belief that *Micrococcus ovalis* and its variants are normal intestinal microbes of the obligate, or nearly obligate lactic acid forming type. Their restriction to the upper segments of the intestinal tract in the nursling, and artificially fed child, is not wholly clear, although current information justifies this statement. In the adult, however, the duodenal habitat can be definitely associated with the dietary status of the host. In this region, but not necessarily below, available carbohydrate is practically

¹⁷ Herter and Kendall: Jour. Biol. Chem., 1908, 5, p. 293; Rahe: Jour. Infect. Dis., 1915, 16, p. 210.

always present if the host is enjoying a mixed diet. The relatively rapid absorption of carbohydrate from the lower levels of the intestinal tract results as a rule in a protein-rich, carbohydrate-free intestinal content, which is unfavorable for the development of the enterococcus.

BACILLUS BIFIDUS

LXXI. STUDIES IN BACTERIAL METABOLISM

ARTHUR ISAAC KENDALL AND REBA CORDELIA HANER

From the Department of Bacteriology and Patten Research Foundation, Northwestern University Medical School, Chicago, Ill.

The examination of a gram-stained specimen of feces from a normal nursingling will usually reveal a multitude of relatively long, slender bacilli, many of them slightly curved, together with a much smaller sprinkling of oval cocci usually occurring in pairs, all retaining the deep blue stain; as well as a few short, gram-negative bacilli and an occasional large, thick bacillus (fig. 10).

The cocci and gram-negative bacilli may be dismissed with a word—they are respectively *Micrococcus ovalis*, or, as it is more commonly referred to, the enterococcus,¹ and members of the colon bacillus group. The long, thin rods, which frequently exhibit irregularities in staining, are for the most part *B. bifidus*.

The morphology of this organism, so characteristic of the normal intestinal flora of the nursingling, together with its irregularly staining involution forms, was accurately described by Escherich² in 1886. This careful observer was unable to induce the organism to grow in any of his cultural mediums, but he did not fail to realize his lack of success, which he discusses in his monograph, at some length.

One of his pupils³ attempted to show that this gram-positive, rod-shaped microbe which was so prominently represented in the nursingling flora, was a colon bacillus. He believed the bacillus acquired the property of retaining the Gram stain during its sojourn within the infantile alimentary tract, and he records experiments purporting to substantiate this view; but it is now known that this surmise was incorrect.

It remained for Tissier⁴ to isolate the microbe, and to describe certain of its peculiar morphologic characteristics, particularly the cleft, or bifid ends, which appear so frequently when it is cultivated outside the human environment (figs. 4, 8 and 9). *B. bifidus*, or *B. bifidus* com-

Received for publication, April 1, 1924.

¹ Kendall and Haner: Jour. Infect. Dis., 1924, 34, p. 5.

² Darmbakterien des Säuglings, 1886.

³ Schmidt, A.: Wien, klin. Wchnschr., 1892, p. 643. Schmidt and Strassburger: Die Faeces des Menschen, 1905, p. 270.

⁴ Recherches sur la flore intestinale des nourrissons, 1900, p. 85.

munis as it was originally designated by Tissier, was found to be an obligate anaerobe. It grows slowly, especially during the first generations outside the human environment. Colonies are rather readily procurable, however, in anaerobic solid mediums containing lactose, after 2 or 3 days' incubation, and subcultures grow somewhat more rapidly.

Carbohydrates, especially lactose, are essential for its growth, and herein lies one of the major difficulties attending its isolation; usually enough colon bacilli, *Bacillus lactis aerogenes* or other aerogenic organisms are present in the nursing dejecta to produce considerable amounts of gas which fragment the medium and cause confluence of colonies, thus interfering with isolation. Serial dilutions of proper specimens, set up as shake cultures in deep lactose agar tubes, are usually successful, especially in the last tubes where but few colonies are present. The more rapidly growing colonies that appear during the first 36 to 48 hours of incubation should be marked and rejected. Those that develop later within the depths of the medium offer considerable prospects of success.

The Gram stained smears prepared from such colonies reveal bacilli exhibiting considerable pleomorphism; 3 principal morphologic types of organisms are clearly discernible. A few gram-positive rods, thin, frequently slightly curved, are reminiscent of the characteristic bacilli found in the original sample of feces (figs. 1 and 7). Some have distinctly enlarged or clubbed ends (fig. 5). A majority of the organisms are either somewhat stouter, with bifid ends, or semivacuolated (figs. 2, 3, 4, 6 and 8). The greater portion of the vacuolated microbe takes the negative stain, leaving a smaller deeply colored blue area. This appearance is quite characteristic (figs. 2, 3 and 6), and does not seem to be exhibited by any microbe thus far described.

Both the bifurcated and vacuolated forms of *B. bifidus* are presumably involution forms, although this view cannot be too definitely defended, as organisms taken from mediums in which carbohydrates are excluded are of the solid bacillary type (figs. 1 and 7), notwithstanding that development is much less luxuriant in these sugar-free cultures.

This departure in morphology from the true bacillary form, which *B. bifidus* exhibits in artificial mediums containing carbohydrate, and so readily recognized by simple staining methods, combined with the obvious difficulties of isolation in pure culture, has doubtless been responsible in no inconsiderable degree for the paucity of information about its chemistry and general cultural reactions.

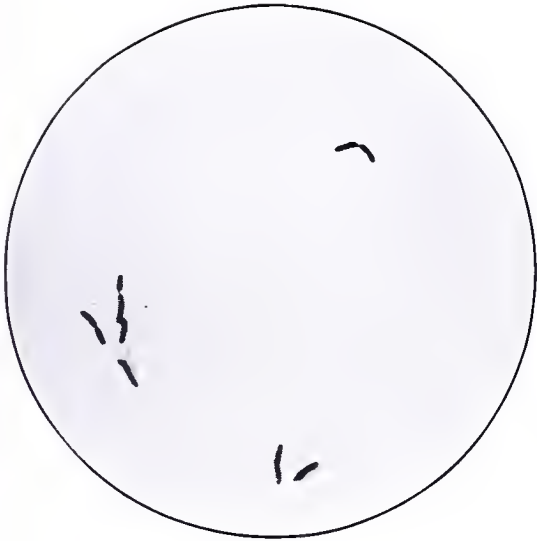


Figure 1

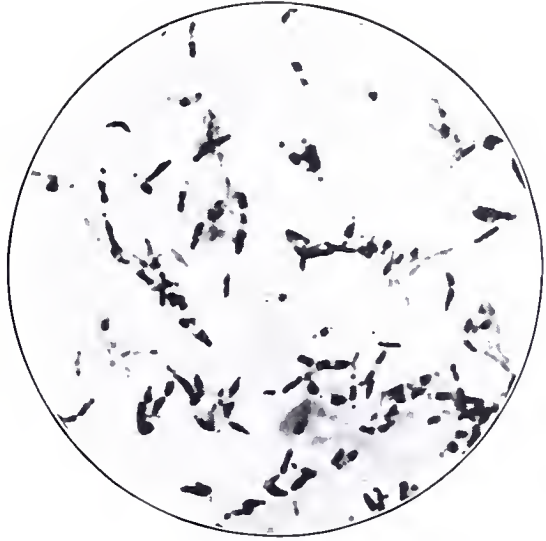


Figure 2

Fig. 1.—*Bacillus bifidus*, $\times 1,200$. Plain broth culture, 72 hours. Slightly curved, solidly staining bacilli.

Fig. 2.—*Bacillus bifidus*, $\times 1,200$. Lactose infusion broth, 72 hours. Irregularly staining bacilli.

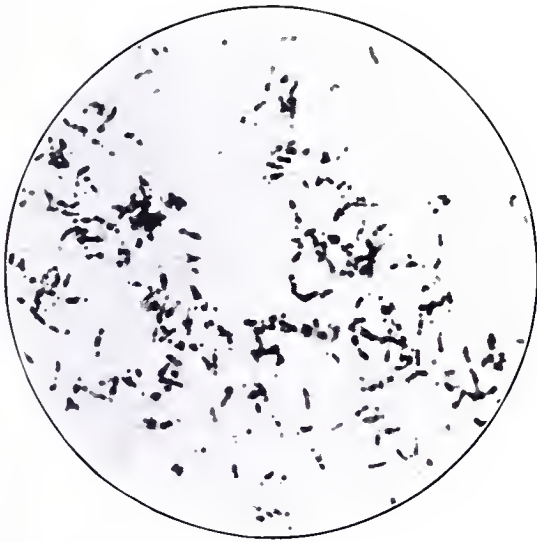


Figure 3

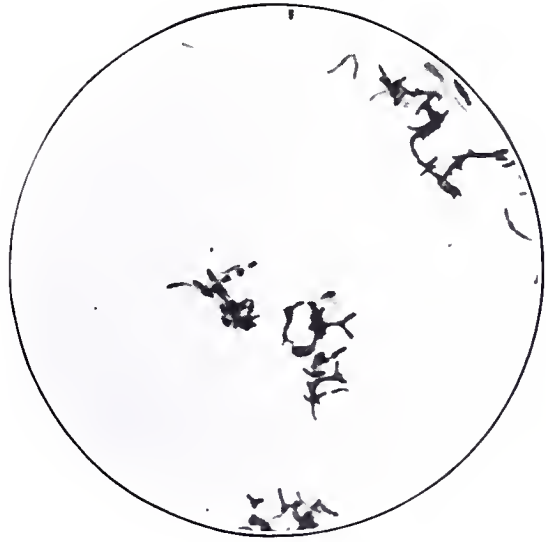


Figure 4

Fig. 3.—*Bacillus bifidus*, $\times 1,200$. Milk culture, 72 hours. Solid and irregularly staining bacilli.

Fig. 4.—*Bacillus bifidus*, $\times 1,200$. Lactose infusion culture, 48 hours. Well defined bifid organisms.

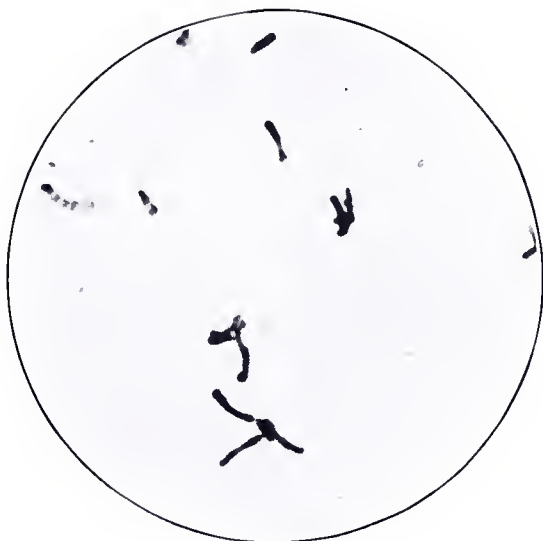


Figure 5



Figure 6

Fig. 5.—*Bacillus bifidus*, $\times 1,200$. Lactose infusion broth, 72 hours. Bacilli with well defined clubbed ends.

Fig. 6.—*Bacillus bifidus*, $\times 1,200$. Lactose infusion culture, 72 hours. Bifid, irregularly staining bacilli.

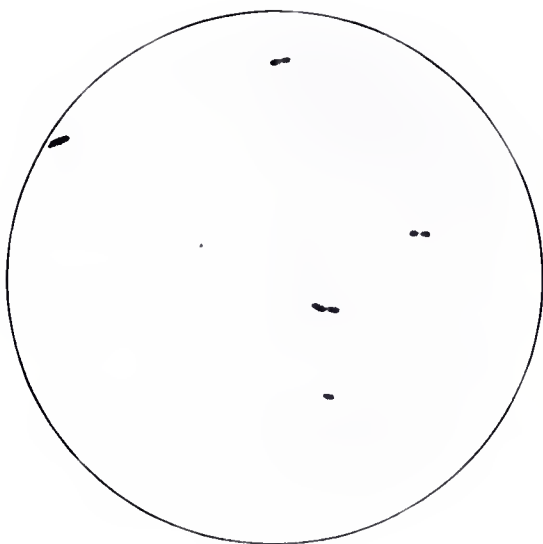


Figure 7

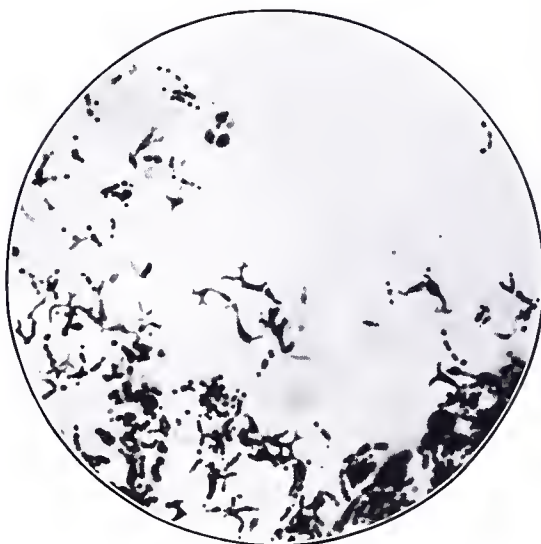


Figure 8

Fig. 7.—*Bacillus bifidus*, $\times 1,200$. Plain broth culture, 72 hours. Ovoid, paired bacilli.

Fig. 8.—*Bacillus bifidus*, $\times 1,200$. Lactose broth culture, 72 hours. Secondary branching and irregularly shaped bacilli.

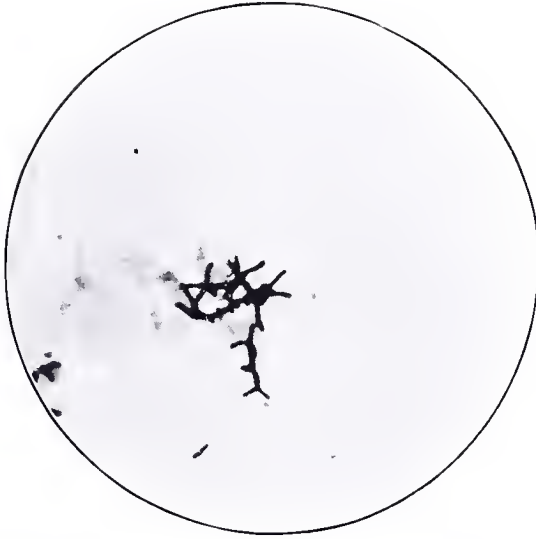


Fig. 9.—*Bacillus bifidus*, $\times 1,200$. Lactose broth culture, 72 hours. Secondary branching of bifidi.

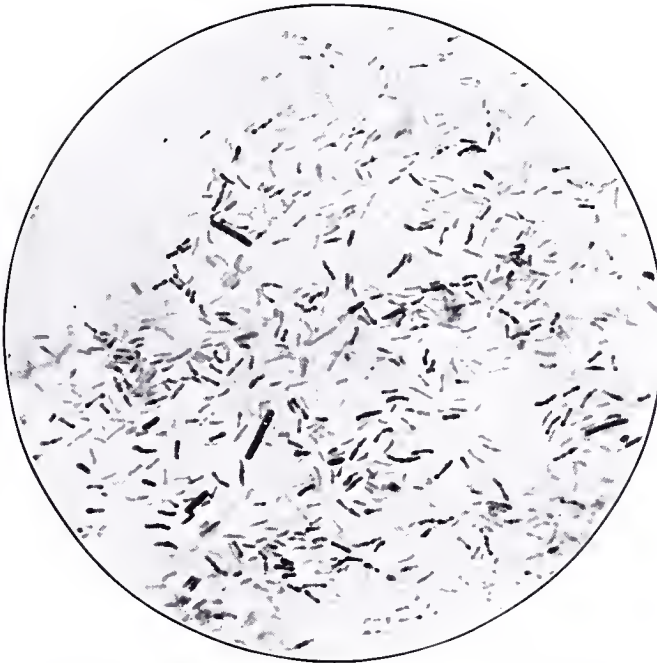


Fig. 10.—Gram stained smear, infant's feces, $\times 1,200$. Predominance of slightly curved, slender rods. Some irregularly staining forms. Three elements of *Bacillus mesentericus*. Few *Micrococcus ovalis*.

The monotony of the normal nursing diet (breast fed), the predominance of the gram-positive bacilli of the bifidus type, and the continuous lactic acid formation in the nursing intestinal tract by these organisms, appear to be three cardinal factors in Nature's plan for shielding the vulnerable alimentary canal of the young child from the assaults of exogenous microbes. As this lactic acid protection has proved to be successful in countless generations of infants, and as the conditions are presumably those on which lactic acid therapy must be planned to be successful in adults, additional information about *B. bifidus* is desirable, especially with reference to its chemistry and general cultural characteristics.

B. bifidus has thus far eluded detection in nature, outside the human body. In this respect it is similar to two other organisms characteristic of very young children, *Micrococcus ovalis*¹ and *Bacillus acidophilus*,⁵ although the latter microbes are also found in the alimentary tracts of older persons and may be identified in sewage at times.

Cultures of *B. bifidus* are not current among laboratory stocks; hence one of us (Haner) isolated several strains from the feces of normal nurslings. These strains were isolated within a short space of time from the dejecta of normal nurslings in the service of Dr. Charles Burt Reed, of Wesley Memorial Hospital, to whom we are indebted for many courtesies. Among these strains, 3 rather persistent morphologic types appeared. The differences were on the whole quantitative, rather than qualitative, but of sufficient magnitude to arrest attention. Figures 1-9, all of the same magnification, illustrate these peculiarities clearly.

There was nothing in the uneventful history of these infants which would afford any explanation for these differences. In the absence of definite information to the contrary, they may be regarded as naturally occurring variants of a fairly fixed fundamental type. The fermentation reactions of these strains follow:

<i>B. bifidus</i>	Glucose	Levulose	Mannose	Galactose	Mannitol	Sorbitol	Dulcitol	Lactose	Saccharose	Dextrin	Milk	No. Cultures Isolated
Type I.....	+	+	+	+	—	—	—	+	+	—	C	4
Type Ia.....	+	+	+	+	—	—	—	+	—	—	C	1
Type II.....	+	+	+	+	—	—	—	+	+	+	C	3
Type III.....	+	+	+	+	+	+	—	+	+	+	C	2

+ indicates fermentation; —, no fermentation, and C, coagulation of milk. None of the types liquefy gelatin.

⁵ Kendall and Haner: Jour. Infect. Dis., 1924, 34, p. 28.

DISCUSSION

The fermentation of the hexose sugars and the inability of strains I and II to utilize the hexose alcohols for energy are not distinctive reactions for bacilli of the obligately fermentative type. Lactose, as might confidently be expected from the diet of the nursling, is readily utilized both in lactose broth and in milk. Saccharose is somewhat less readily fermented than lactose, and this may have some relationship to the well-known disappearance of bifidi from the feces of young children

TABLE 1
TITRATABLE ACIDITIES. QUANTITATIVE FERMENTATION REACTIONS OF *B. BIFIDUS* TYPES

<i>B. bifidus</i>	Day	Plain Gelatin	Glucose Gelatin	Lactose Gelatin	Sucrose Gelatin	Dextrin Gelatin	Milk
Control....	..	+0.6	+ 0.6	+ 0.6	+ 0.6	+0.6	+ 1.7
Type I....	1	+0.6	+ 7.9	+ 7.5	+ 1.4	+ 2.5
	3	+0.8	+11.5	+10.8	+ 1.7	+0.8	+ 9.0
	6	+1.0	+11.2	+10.7	+ 2.4	+14.4
	10	+0.8	+11.0	+10.5	+ 3.5	+1.0	+14.2
Control....	..	+0.7	+ 0.8	+ 0.7	+ 0.7	+0.7	+ 1.6
Type Ia....	1	+0.8	+ 6.7	+ 6.7	+ 1.5	+1.2	+ 2.0
	3	+1.0	+12.6	+11.6	+11.1	+1.7	+ 2.5
	7	+1.3	+15.1	+12.4	+12.8	+1.7	+ 3.5
	10	+1.2	+15.1	+12.6	+12.8	+1.8	+ 4.4
	14	+1.0	+14.6	+14.4	+13.1	+2.8	+ 6.2
Control....	..	+0.3	+ 0.3	+ 0.3	+ 0.3	+0.2	+ 1.7
Type II....	1	+0.4	+ 2.8	+10.4	+ 1.1	+1.2	+ 4.9
	3	+0.4	+ 9.1	+10.8	+12.6	+6.7	+ 6.6
	6	+0.3	+ 9.4	+11.7	+12.5	+8.4	+ 9.2
	10	+0.5	+10.6	+17.7	+13.0	+7.1	+10.7
	14	+0.5	+ 9.6	+18.6	+6.2	+11.6
Control....	..	+0.5	+ 0.6	+ 0.5	+ 0.6	+0.6	+ 1.9
Type III....	3	+10.2	+12.6	+ 5.9	+7.1	+ 5.6
	7	+0.5	+11.1	+16.4	+ 8.3	+6.6	+ 9.7
Type III....	3	+0.6	+ 9.9	+10.9	+ 2.6	+13.4
	7	+0.5	+12.7	+10.1	+ 6.4	+6.6	+15.5

The "protein error" induced by the gelatin in these mediums precluded the determination of the hydrogen-ion changes. They may be estimated, however, with considerable readiness by correcting the normality of the acid by the dissociation constant for lactic acid.

at the end of the nursling period. It is worthy of note that the more commonly occurring type I fails to utilize dextrin. *B. acidophilus*, which tends to supplant *B. bifidus* in the alimentary canals of young children artificially fed, appears to utilize the glucose glucoside configuration with comparative readiness.⁵

Strain 3 is more versatile in its fermentation reactions. It can utilize the hexose alcohols, mannitol and sorbitol, in addition to the corresponding hexose aldehydes. One strain of *B. acidophilus* exhibited a similar adaptability.⁵

Too much importance cannot be attached to qualitative fermentation reactions, however, even though they suggest in no uncertain terms real

differences in the protoplasmic configurations. Biologic science has as yet not advanced far enough to comprehend the intimate stereochemical reaction of living cells.

The quantitative changes in titratable acidity in certain mediums having dietary significance are shown in table 1:

Much more significant in the light of current knowledge is the nitrogenous metabolism of the intestinal microbes. They are in competition with the host for nutrition, and while even trillions of bacteria do not abstract very much nitrogen from the common intestinal contents to incorporate in their bodies, it is of much consequence to their host whether or not they form products that may be inimical.⁶

TABLE 2
BACTERIAL METABOLISM OF *B. BIFIDUS*, TYPE I

Mg. per 100 C c.	Day	Control Gelatin Broth	Gelatin Broth	Control Glucose Gelatin	Glucose Gelatin	Control Lactose Gelatin	Lactose Gelatin	Control Milk	Milk
Total nitrogen.....	1	1,057	1,057	1,036	1,036	1,036	1,036	427	427
Protein nitrogen.....		840	847	833	819	819	826	385	378
Nonprotein nitrogen		217	210	203	217	217	210	42	49
Polypeptid nitrogen.		156.8	147.0	143.5	156.1	159.6	152.6	19.6	23.8
Amino nitrogen.....		41.3	45.5	42.7	46.2	39.9	43.4	19.6	22.4
Ammonia nitrogen..		18.9	17.5	16.8	14.7	17.5	14.0	2.8	2.8
Total nitrogen.....	3	1,057	1,057	1,036	1,036	1,036	1,036	427	427
Protein nitrogen.....		840	840	833	819	819	826	385	364
Nonprotein nitrogen		217	217	203	217	217	210	42	63
Polypeptid nitrogen.		156.8	155.4	143.5	158.2	159.6	149.8	19.6	42.7
Amino nitrogen.....		41.3	43.4	42.7	45.5	39.9	46.9	19.6	20.3
Ammonia nitrogen..		18.9	18.2	16.8	13.3	17.5	13.3	2.8	0.0
Total nitrogen.....	6	1,057	1,057	1,036	1,036	1,036	1,036	427	427
Protein nitrogen.....		840	854	833	819	819	854	385	364
Nonprotein nitrogen		217	203	203	217	217	182	42	63
Polypeptid nitrogen.		156.8	140.0	143.5	153.3	159.6	121.8	19.6	42.0
Amino nitrogen.....		41.3	44.1	42.7	49.0	39.9	45.5	19.6	20.3
Ammonia nitrogen..		18.9	18.9	16.8	14.7	17.5	14.7	2.8	0.7
Total nitrogen.....	10	1,057	1,057	1,036	1,036	1,036	1,036	427	427
Protein nitrogen.....		840	854	833	875	819	861	385	364
Nonprotein nitrogen		217	203	203	161	217	175	42	63
Polypeptid nitrogen.		156.8	140.0	143.5	95.9	159.6	112.7	19.6	42
Amino nitrogen.....		41.3	43.4	42.7	49.7	39.9	46.2	19.6	18.9
Ammonia nitrogen..		18.9	19.6	16.8	15.4	17.5	16.1	2.8	3.1

With this objective in view, a study of the nitrogenous metabolism was made of 3 strains of *B. bifidus*, using the methods previously described.⁷ Plain gelatin, glucose and lactose broths containing 5 per cent of gelatin and milk were selected as representative mediums. The cultures were grown in anaerobic flasks of special design.⁸

The following table shows the nitrogenous change induced in these mediums at varying intervals. The results are expressed in milligrams of nitrogen per 100 c c. of culture medium.

⁶ Kendall: *Physiol. Abst.*, 1923, 3, p. 438.

⁷ Kendall: *Jour. Infect. Dis.*, 1922, 30, p. 211.

⁸ Kendall, Cook and Ryan: *Jour. Infect. Dis.*, 1921, 29, p. 227.

TABLE 3
BACTERIAL METABOLISM OF B. BIFIDUS, TYPE II

Mg. per 100 C c.	Day	Control Gelatin Broth	Gelatin Broth	Control Glucose Gelatin	Glucose Gelatin	Control Lactose Gelatin	Lactose Gelatin	Control Milk	Milk
Total nitrogen.....	1	1,008	1,008	945	945	945	945	448	448
Protein nitrogen.....		805	784	749	749	749	735	399	399
Nonprotein nitrogen		203	224	196	196	196	210	49	49
Polypeptid nitrogen.		144.2	165.2	140.0	137.2	140.0	153.3	25.2	23.8
Amino nitrogen.....		40.6	41.3	37.8	42.7	37.8	41.3	18.2	21.7
Ammonia nitrogen..		18.2	17.5	18.2	16.1	18.2	15.4	5.6	3.5
Total nitrogen.....	3	1,008	1,008	945	945	945	945	448	448
Protein nitrogen.....		805	805	749	749	749	735	399	385
Nonprotein nitrogen		203	203	196	196	196	210	49	63
Polypeptid nitrogen.		144.2	141.4	140	135.1	140.0	151.2	25.2	37.8
Amino nitrogen.....		40.6	43.4	37.8	44.1	37.8	42.0	18.2	19.6
Ammonia nitrogen..		18.2	18.2	18.2	16.8	18.2	16.8	5.6	5.6
Total nitrogen.....	7	1,008	1,008	945	945	945	945	448	448
Protein nitrogen.....		805	812	749	763	749	763	399	385
Nonprotein nitrogen		203	196	196	182	196	182	49	63
Polypeptid nitrogen.		144.2	133.7	140	123.2	140.0	125.3	25.2	36.4
Amino nitrogen.....		40.6	44.1	37.8	42.7	37.8	40.6	18.2	21.0
Ammonia nitrogen..		18.2	18.2	18.2	16.1	18.2	16.1	5.6	5.6
Total nitrogen.....	10	1,008	1,008	945	945	945	945	448	448
Protein nitrogen.....		805	826	749	777	749	777	399	399
Nonprotein nitrogen		203	182	196	168	196	168	49	49
Polypeptid nitrogen.		144.2	121.4	140	109.2	140	109.2	25.2	25.2
Amino nitrogen.....		40.6	43.4	37.8	42.0	37.8	42.0	18.2	16.8
Ammonia nitrogen..		18.2	18.2	18.2	16.8	18.2	16.8	5.6	7.0
Total nitrogen.....	14	1,008	1,008	945	945	945	945	448	448
Protein nitrogen.....		805	805	749	742	749	770	399	406
Nonprotein nitrogen		203	203	196	203	196	175	49	42
Polypeptid nitrogen.		144.2	137.2	140.0	140	140.0	114.8	25.2	17.5
Amino nitrogen.....		40.6	45.5	37.8	44.8	37.8	43.4	18.2	18.9
Ammonia nitrogen..		18.2	20.3	18.2	18.2	18.2	16.8	5.6	5.6

TABLE 4
BACTERIAL METABOLISM OF B. BIFIDUS, TYPE III

Mg. per 100 C c.	Day	Control Gelatin Broth	Gelatin Broth	Control Glucose Gelatin	Glucose Gelatin	Control Lactose Gelatin	Lactose Gelatin	Control Milk	Milk
Total nitrogen.....	1	966	966	882	882	882	882	413	413
Protein nitrogen.....		777	777	714	693	728	728	371	371
Nonprotein nitrogen		189	189	168	189	154	154	42	42
Polypeptid nitrogen.		127.4	128.8	108.5	131.6	94.5	95.2	16.8	19.6
Amino nitrogen.....		43.4	42.0	41.3	39.2	42.0	43.4	18.9	17.5
Ammonia nitrogen..		18.2	18.2	18.2	18.2	17.5	15.4	6.3	4.9
Total nitrogen.....	3	966	966	882	882	882	882	413	413
Protein nitrogen.....		777	763	714	693	728	707	371	371
Nonprotein nitrogen		189	203	168	189	154	175	42	42
Polypeptid nitrogen.		127.4	141.4	108.5	128.1	94.5	116.2	16.8	22.4
Amino nitrogen.....		43.4	42.0	41.3	43.4	42.0	42.7	18.9	16.1
Ammonia nitrogen..		18.2	19.6	18.2	17.5	17.5	16.1	6.3	3.5
Total nitrogen.....	6	966	966	882	882	882	882	413	413
Protein nitrogen.....		777	763	714	686	728	700	371	371
Nonprotein nitrogen		189	203	168	196	154	182	42	42
Polypeptid nitrogen.		127.4	138.6	108.5	135.1	94.5	122.5	16.8	21.0
Amino nitrogen.....		43.4	42.7	41.3	41.3	42.0	41.3	18.9	17.5
Ammonia nitrogen..		18.2	21.7	18.2	18.9	17.5	18.2	6.3	3.5
Total nitrogen.....	10	966	966	882	882	882	882	413	413
Protein nitrogen.....		777	770	714	686	728	714	371	378
Nonprotein nitrogen		189	196	168	196	154	168	42	35
Polypeptid nitrogen.		127.4	133.0	108.5	135.8	94.5	109.2	16.8	14
Amino nitrogen.....		43.4	42.0	41.3	42.7	42.0	42.7	18.9	18.2
Ammonia nitrogen..		18.2	21.0	18.2	17.5	17.5	16.1	6.3	2.8
Total nitrogen.....	14	966	966	882	882	882	882	413	413
Protein nitrogen.....		777	...	714	...	728	...	371	...
Nonprotein nitrogen		189	...	168	...	154	...	42	...
Polypeptid nitrogen.		127.4	...	108.5	...	94.5	...	16.8	25.2
Amino nitrogen.....		43.4	45.5	41.3	44.1	42.0	45.5	18.9	23.1
Ammonia nitrogen..		18.2	20.3	18.2	18.2	17.5	14.7	6.3	2.1

DISCUSSION

The various strains of *B. bifidus* are virtually alike in that their reactions on the protein mediums are so slight as to be almost undetectable with the methods customarily employed. Not only is the quantitative change in the protein constituent very small, but also no aromatic substances of the indol, skatol, histamine and cresol group could be detected by qualitative tests. This is of some significance. *B. bifidus*, judging from the strains studied, is practically without measurable action on proteins. It grows feebly in protein mediums, even those reinforced with meat or similar nearly native protein, if carbohydrate is withheld. The organism appears to be a member of the obligately fermentative type.

This probably explains, in part at least, the absence of bifidi in the intestinal contents of adults, for whom carbohydrates form a relatively small fraction of the normal diet. The members of the bifidus group tend to localize in the upper levels of the large intestine, even in the nursing. This is presumably an additional factor tending to restrict the growth of the organisms in adults, because in them the large intestine is usually quite free from lactose, the most favorable carbohydrate for the luxuriant growth of bifidi.

A study of the feces of several hundreds of adults has shown that *B. bifidus* is of comparatively rare occurrence. One patient, a heavy drinker of milk, did harbor considerable numbers of bifidi habitually. A few bifidi may be found from time to time to time in adults living on a mixed diet. Heavy lactose diets, as for example that frequently administered to patients with typhoid and dysentery,⁹ not uncommonly lead to the appearance of *B. bifidus* in moderate numbers, however. This leads to speculation concerning the origin of bifidus in such cases, none being detected in normal feces of adults. Many surmises have been indulged in, but no known fact throws light on this peculiar appearance of the organism when dietary conditions parallel those of the normal nursing.

The suggestion has been made that *B. bifidus* represents what may be designated a "fermentative phase" of some more common and versatile intestinal microbe, but at present there is no conclusive evidence that any bacterium shifts its chemical architecture, its enzyme equipment, and its general chemical behavior to such a degree as to simulate widely different types with no discernible transitional stages.

⁹ Kendall: Boston Med. & Surg. Jour., 1910, 163, p. 398; 1911, 164, p. 288; 1913, 169, p. 754; Torrey: Jour. Infect. Dis., 1915, 16, p. 72.

That intestinal conditions may impress unexpected characteristics, as, for example, tolerance to organic acids on certain bacteria, seems to have some basis in fact,¹⁰ but in such instances there is no discernible violent shifting of the characteristics relied on for identification. Qualitatively the microbes seem to be as they were originally, and the quantitative departure from type soon reverts to the normal if the microbes exhibiting them are placed in a normal culture environment.

It should be mentioned in passing that bacteria would appear to offer unusual opportunities for the study of fundamental factors in continuous and discontinuous variations. The bacteria are without sex, which would seem to eliminate certain remote hereditary tendencies which may be accentuated through the dual parentage. Also, successive generations appear at short intervals. Hundreds of generations may be passed in review in a week.

Ordinarily the preservation of stock cultures for purposes of instruction and investigation is carried with the definite expectation of preserving, not creating, characteristics. Possible variants and mutants are overlooked or discarded.

The spontaneous appearance of mucin-like substance in stock cultures of tubercle, glanders, and other bacteria, may be cited as a possible instance of the appearance of chemical variants.¹¹ The loss of characteristics, such as the gradual disappearance of the protein liquefying enzyme of *B. proteus*, and its loss of saccharose fermenting power is presumably not a true instance of variation or mutation.¹²

SUMMARY

B. bifidus, as its variants, is an anaerobic organism exhibiting distinct tendencies toward pleomorphism in artificial cultural mediums, especially lactose. It is inert, both culturally and chemically, in mediums containing proteins or protein derivatives, but no utilizable carbohydrate. In the presence of the latter, however, it produces considerable amounts of organic acids, chiefly lactic.

It has no discernible action on amino acids containing aromatic nuclei, which makes it theoretically at least a suitable microbe for intestinal implantation and dieto therapy. Its occurrence in the intestinal contents and feces of normal nurslings is significant in this respect,

¹⁰ Kendall: Jour. Med. Res., 1910, 22, p. 153.

¹¹ Smith, Theobald: Trans. First Annual Meet. Nat'l. Assn. Study of Tuberc. Weleminsky: Wien. klin. Wchnschr., 1912, 25, p. 614. Kendall, Day and Walker: Jour. Infect. Dis., 1914, 15, p. 428.

¹² Kendall, Cheetham and Hamilton: Jour. Infect. Dis., 1922, 30, p. 251.

but the difficulties of isolation and cultivation do not characterize it as an economic competitor of *Bacillus acidophilus*. From the chemical point of view, the disproportionate action of the bifidus group on the galactose glucoside configuration offers a rather striking contrast to the members of the acidophilus group, which seem to utilize the glucose glucoside configuration to better advantage.

BACILLUS ACIDOPHILUS

LXXII. STUDIES IN BACTERIAL METABOLISM

ARTHUR ISAAC KENDALL AND REBA CORDELIA HANER

From Department of Bacteriology and Patten Research Foundation, Northwestern University Medical School, Chicago, Ill.

One of the romances of bacteriology is that interesting but somewhat speculative volume written by Metchnikoff, entitled "The Prolongation of Life."¹ In this book will be found a discussion of premature senility and autointoxication, together with the development of the theory that these manifestations are related to the activity of putrefactive anaerobic bacteria in the lower levels of the intestinal tract. Metchnikoff did not cease his labors with the pronouncement of this theory, which was not entirely novel at the time he began his investigations, but attacked boldly the much more difficult task of discovering remedial measures. He realized that cures could not be hoped for in the light of the damage already done in such cases.

Germ-free alimentary tracts are impossible of realization, except under circumstances wholly beyond livable conditions, and even temporary sterilization of the intestinal contents is not feasible with any disinfectant known to medicine.²

Another line of approach, that of replacing malignant microbes with good germs, presented itself, and it was to this possibility that Metchnikoff devoted himself. Bacterial implantation in the alimentary canal was not an entirely novel procedure—Herter³ had already introduced colon bacilli into the intestinal tract for therapeutic purposes—but the idea of definitely supplanting resident bacteria exhibiting unfavorable characteristics by microbes alien to the intestinal tract, possessed of desirable qualities, was a new departure.

Attention is directed at this point to a wide divergence between the Herter and Metchnikoff points of view. Herter reintroduced a normal intestinal organism, *Bacillus coli*; Metchnikoff, as will be shown later, selected a microbe wholly foreign to the intestinal flora, for his experiments.

Received for publication April 1, 1924.

¹ English translation.

² Kendall: Jour. Med. Res., 1911, 25, p. 117.

³ Brit. Med. Jour., 1897, 2, p. 1847.

In casting about for suitable cultures, Metchnikoff's attention was directed toward the Bulgarians, who appeared at that time at least to live to a ripe old age. Autointoxication and premature senility were said to be uncommon complications in the otherwise placid Bulgarian existence.

Soured milk is an important item in the diet of the Bulgarian peasant. Indeed, the milk of his herds is an irreplaceable source of nutriment. The ice man never visits Bulgaria, and the herdsmen have learned, even as the nomads of the oriental deserts have learned, to conserve the precious, but unstable lacteal fluid from putrefying by the addition of "starters" to it as soon as it is drawn. Of course the Bulgarian does not realize that he is availing himself of a practical bacteriologic principle when he throws his lump of casein into the offering of his herd.

This lump or ball of casein, which contains an impure culture of active lactic acid bacilli, is an invaluable part of the Bulgarian Lares and Penates. Between milkings, it is kept in a dark place, wrapped to prevent evaporation; cast into freshly drawn milk, it seeds the entire volume with rapidly growing lactose fermenting microbes, which quickly raise the acidity to a point at which putrefaction organisms cannot develop. When the milk is thoroughly soured, the casein ball, rejuvenated meanwhile, is withdrawn and kept until the next milking.

The essential fact to be recognized is that the cycle of the lactic acid bacilli in the Bulgarian complex is from milk to milk; in no instance do the bacteria circulate between the alimentary canal of the peasant and the milk pail.

These lactic acid bacteria were the ones selected by Metchnikoff for his implantation experiments. It is quite clear that they are milk parasites and in no sense intestinal parasites. Apparently they never have been exposed to the complex conditions obtaining in the human alimentary canal.

Bacteria parasitized in milk do not seem to possess the same adaptability to the intestinal habitat as do the normal intestinal parasites, even though both types produce much lactic acid in milk outside the human body. It is not surprising, therefore, to find that *B. bulgaricus*—for such was the name conferred on this microbe by Metchnikoff—does not occur in the feces of the Bulgarian, nor are the normal lactic acid bacilli of the Bulgarian feces, so as far as available evidence shows, found in the Bulgarian milk pail.

Attempts to demonstrate viable *B. bulgaricus* in the feces of human subjects, even after the prolonged administration of milk soured by Bulgarian bacilli in pure culture, have failed.⁴

No inconsiderable part of the opposition to lactic acid bacillus therapy which has grown in the last few years, however, is attributable, not to Metchnikoff, whose intuitive genius saw the possibilities of this procedure even if he did miss one salient detail, but to the exploitation of the idea which Metchnikoff's well deserved fame popularized.

Many varieties of pills, tablets, candies, and vials, purporting to contain live and active cultures of *B. bulgaricus*, or some other microbe just as desirable, have been sold to the uninformed public both in this country and abroad. It cannot be said that these preparations did harm by design, but, alas, they did little or no good by mistake. Many of these pills and tablets and vial cultures have been found to contain so few live organisms that literally hundreds of them individually or collectively would fail to yield a satisfactory number of bacteria. If by chance such preparations do contain modest numbers of bacteria, the administration of them is inevitably doomed to failure, because no provision is made for the proper nutrition of the bacteria in the alimentary tract after they are swallowed. Lactic acid bacteria require suitable carbohydrate in constant, minimal amounts, in order to produce their characteristic product. Furthermore, the microbes are not intestinal parasites.

This is not the time or place to discuss the intestinal implantation aspect of lactic acid therapy, but a few cardinal principles must be observed to lay the foundation for success. These are:

1. A microbic culture suitable for, and acclimatized to, intestinal conditions in man.⁵

2. A properly modified diet to reduce protein residua in the human alimentary canal to relatively small amounts, and a carbohydrate regimen so adjusted as to provide a continuous supply of this absolutely necessary type of foodstuff throughout the small and large intestines, to at least the sigmoid flexure.

3. The absence of a contraindication to a carbohydrate rich diet.⁶

The organisms best adapted to intestinal implantation and intestinal lactic acid therapy are of two types, both potent lactic acid formers found naturally in the alimentary canal. One of these, *B. bifidus*, is an anaer-

⁴ Herter and Kendall: Jour. Biol. Chem., 1908, 5, p. 293. Rahe: Jour. Infect. Dis., 1915, 16, p. 210.

⁵ Kendall: Am. Jour. Med. Sc., 1918, 156, p. 157.

⁶ Kendall and Smith: Boston Med. and Surg. Jour., 1911, 164, p. 306.

robe, characteristically present in the intestinal contents;⁷ the other, *B. acidophilus*, is a common inhabitant of the intestinal tracts of artificially fed children and of many adults. It is even detectable in sewage.⁸ It is to the latter type, or rather group, of microbes, of which *B. acidophilus* is the best known, that this discussion specifically relates.

B. acidophilus was described independently by Finkelstein⁹ and Moro¹⁰ as an organism occurring normally in the intestinal tracts of artificially nourished infants. It is also detectable in smaller numbers in the dejecta of breast-fed babies. The microbe is less commonly found in adults, although some persons harbor relatively luxuriant growths of *B. acidophilus*.

Even the most casual perusal of the earlier studies of *B. acidophilus* will reveal the paucity of definite knowledge about the microbe. This is due chiefly to a cultural peculiarity of the organism which renders its cultivation apparently a simple procedure. Both Finkelstein and Moro obtained growths of *B. acidophilus* in mediums purposely rendered so acid with organic acids¹¹ that a majority, and indeed nearly all other, bacteria would not develop. It was from this striking viability in the presence of organic acids that the term "acidophilus" was coined by Moro. It is now rather generally conceded that the term "acid-loving" is a misnomer, and the group of organisms to which *B. acidophilus* belongs is more properly designated the "aciduric bacteria."⁸ The tolerance which acidophili manifest toward organic acids is an incident, not a characteristic.¹² As a matter of fact, the ability of acidophili to grow in acid mediums and their tolerance to organic acid acidities are subject to rather wide quantitative variations, both among different strains of the organism and in the same strain at different times and under varying conditions. As the recognition of the organism, rather than an inquiry into its antecedents, was the objective of the earlier studies, however, this unusual feature of acid tolerance was very useful, and it is not surprising to find that it remained a prominent method for the recognition of the microbe for more than two decades.

Within the last 15 years, a gradual change in method has taken place; the purely morphologic and statistical study of intestinal bacteria is being

⁷ Kendall, Arthur Isaac, and Haner, Reba Cordelia: *Jour. Infect. Dis.*, 1924, 35, p. 16.

⁸ Kendall: *Jour. Med. Res.*, 1910, 22, p. 153. Rahe: *Jour. Infect. Dis.*, 1914, 15, p. 141. Cheplin and Rettger: *Proc. Soc. Exper. Biol. & Med.*, 1920, 17, p. 192. Cannon: *Jour. Infect. Dis.*, 1924, 34, p. 227.

⁹ *Deutsch. med. Wchnschr.*, 1900, 26, p. 263.

¹⁰ *Wien. klin. Wchnschr.*, 1900, 13, p. 114.

¹¹ Mineral acids are not suitable for this purpose.

¹² *Jour. Infect. Dis.*, 1914, 15, p. 141.

supplanted by the contemplation of those factors or elements which determine the occurrence of specific types of microbes in the alimentary tract. The dynamic follows the static in nearly all lines of investigation. Prominent among these newer procedures is the chemistry of microbic activity.¹³

The chemistry of the acidophili does not seem to have been studied hitherto. As these organisms are natural inhabitants of the alimentary tract, occurring under conditions which are normal and, theoretically at least, for the most part desirable, data on the metabolism of the group would seem to have more than theoretical interest.

One of us (Haner) has isolated strains of acidophili from a considerable number of specimens, and thus far 3 rather distinct morphologic types are recognizable among them. Culturally, however, they are almost similar, although the one designated type 3 is somewhat more versatile in its fermentation reactions, utilizing mannitol and sorbitol, which are not utilizable for energy by the other two types.

TABLE 1
FERMENTATION REACTIONS OF *B. ACIDOPHILUS* TYPES

Type	Glucose	Levulose	Mannose	Galactose	Sorbitol	Mannitol	Dulcitol	Maltose	Lactose	Saccharose	Dextrin	Milk	Gelatin Liquefied
1	+	+	+	+	—	—	—	+	+	+	+	C	—
2	+	+	+	+	—	—	—	+	+	+	+	C	—
3	+	+	+	+	+	+	—	+	+	+	+	C	—

+ indicates fermentation; —, no fermentation, except gelatin where — indicates no liquefaction; C, coagulation; 7 day observations.

The 3 acidophilic types mutually convey an impression of a rather vigorous fermentative organism which is capable of utilizing all the common hexose and biose carbohydrates (and dextrin as well) found in the diet of the healthy person. This is of significance in view of the intestinal conditions which prevail in those who harbor acidophili. Almost any regimen which will leave a continuous residuum of carbohydrate at all levels of the intestinal tract as far down as the sigmoid flexure will encourage the growth of the members of the aciduric group. A monotonous breast milk diet, however, as that of the normal nursing, does not usually lead to the aciduric flora; members of the *B. bifidus* group are much more commonly found under these circumstances.¹⁴

¹³ Kendall: *Endocrinology and Metabolism*, 1923, p. 663; *Physiological Reviews*, 1923, 3, p. 438.

¹⁴ Kendall: *Boston Med. & Surg. Jour.*, 1911, 164, p. 288. Kendall and Haner: *Jour. Infect. Dis.*, 1924, 35, p. 16.

It might be inferred, although convincing data are not available, that polymers of glucose and glucose glucosides are somewhat more favorable configurations for the acidophili than the galactose glucoside configuration for energy. The rather sluggish growth which freshly isolated intestinal strains of acidophilus exhibit in milk may be of some significance in this connection.

Type 3 is versatile in that it can derive energy from mannitol and sorbitol. It will be recalled⁷ that a mannitol-sorbitol fermenting strain was discovered in the *B. bifidus* group.

TABLE 2
TABLE SHOWING TITRABLE ACIDITY IN VARIOUS FERMENTATION MEDIUMS AND IN MILK;
REACTIONS OF *B. ACIDOPHILUS*

Reaction, C c. N/1 Acid per 100 C c.	Day	Plain Gelatin	Glucose Gelatin	Laetose Gelatin	Sucrose Gelatin	Dextrin Gelatin	Milk
Control.....		+0.30	+0.30	+0.20	+0.30	+0.30	+1.90
<i>B. acidophilus</i> , Type I.....	1	+0.70	+8.20	+7.30	+8.00	+1.50	+7.10
	3	+0.80	+10.90	+9.20	+9.70	+2.00	+11.20
	6	+0.80	+9.40	+8.60	+9.70	+2.00	+12.70
	10	+0.80	+9.40	+8.60	+9.30	+2.20	+14.00
Control.....		+0.10	+0.30	+0.20	+0.20	+0.20	+1.90
<i>B. acidophilus</i> , Type Ia.....	1	+0.20	+0.60	+0.80	+0.50	+0.50	+3.90
	3	+0.80	+4.70	+6.80	+1.60	+1.00	+13.40
	6	+0.60	+8.90	+9.70	+7.10	+1.00	+20.00
	8	+0.60	+8.90	+9.10	+9.00	+1.20	+20.00
	10	+0.70	+9.00	+10.10	+9.00	+1.80	+21.30
Control.....		+0.20	+0.30	+0.30	+0.30	+0.40	+1.60
<i>B. acidophilus</i> , Type II.....	1	+1.10	+4.80	+3.40	+4.30	+1.40	+2.00
	3	+1.00	+7.90	+7.90	+8.20	+1.60	+4.10
	6	+1.20	+9.10	+8.50	+9.00	+2.30	+5.70
	8	+1.40	+9.20	+9.20	+9.00	+2.50	+6.90
	10	+1.40	+9.30	+8.80	+8.50	+2.60	+7.40
Control.....		+0.70	+0.70	+0.50	+0.60	+0.60	+1.90
<i>B. acidophilus</i> , Type III.....	3	+1.60	+10.90	+5.00	+1.90	+2.90	+8.30
	6	+2.20	+10.40	+5.00	+2.90	+4.10	+11.30
	11	+2.70	+10.30	+5.80	+5.20	+5.30	+12.70

A feature of some interest is the relative amount of acidity produced in fermentation mediums and in milk by these strains. The titratable change is recorded in table 2 as the number of cubic centimeters of normal acid in 100 c c. of medium. It will be noticed that the reaction of control mediums in each instance is slightly but distinctly on the acid side of neutrality. This is important, because many carbohydrates undergo spontaneous change in mediums of even slightly alkaline reaction of such magnitude that the results obtained with them are unreliable.¹⁵

¹⁵ Kendall and Yoshida: *Ibid.*, 1923, 32, pp. 355, 362 and 369.

It will be seen that even in mediums containing protein constituents but no added carbohydrates, there is a slight but unmistakable increase in titratable acidity. The hydrogen-ion concentration of such mediums also increases.¹⁶ It seems probable that the so-called carbohydrate radical of protein may be responsible for this acidity; previous studies have revealed a similar phenomenon, and qualitative tests have indicated a basis for this possibility.¹⁷

Special mention should be made of the change in reaction which occurs in the various milk cultures. These are more marked than the synchronous development of acidity in the various fermentation mediums. Both the rate and the intensity of the acid development varies quite markedly with the culture.

At first sight, it might be assumed that this was an inherent characteristic, peculiar to each strain, and this would be somewhat in accord with the unmistakable variations in acid tolerance of the organisms. This explanation cannot be accepted as an unqualified exposition, however. It is significant, but by no means an invariable coincidence, that the most intense acid producer (type 1a) was cultivated outside the intestinal tract for many months, while the lesser acidogenic type (2) was recently isolated.

Available evidence indicates a resultant between the two factors of inherent acidogenesis and cultural adaptability as being the more probable explanation of these differences.

A careful search in the carbohydrate-free mediums for aromatic derivatives of amino acids that give color reactions—indol, phenols, histamine—failed to reveal the slightest indication that acidophili deaminize or decarboxylize tryptophan, tyrosin, phenyl alanine or histidine. This is of some significance in judging the fitness of *B. acidophilus* for intestinal implantation.

The totality of qualitative information of the *B. acidophilus* group centers around a microbe or microbes which belong to the obligately fermentative type.¹⁸

An interesting and most important practical question presents itself: What changes do members of the aciduric group bring about in food residues of the alimentary canal? Escherich¹⁹ and Salge²⁰ have found

¹⁶ The "protein error" introduced by the addition of gelatin to the mediums is of sufficient magnitude to make hydrogen-ion determinations of insignificant value in this series.

¹⁷ Kendall and Farmer: *Jour. Biol. Chem.*, 1912, 12, p. 215.

¹⁸ Kendall: *Boston Med. & Surg. Jour.*, 1910, 163, p. 322; *Pediatrics*, 1910, 27, p. 613.

¹⁹ *Jahrb. f. Kinderheilk.*, 1900, 52, p. 1.

²⁰ *Die akute Dünndarmkatarrh des Säuglings*, 1906.

acidophilic-like organisms in cases of "Blaue Bazillöse" which suggest that an overgrowth of members of the group may cause unfavorable conditions within the alimentary tract. As these two observations, however, seem to be the only ones recorded, too much attention should not be paid to an unusual occurrence like this.

Obviously, if these microbes are to be implanted in the intestinal tract, in competition with the host for some of the food there, it is of more than academic interest to forecast whether the products of their metabolism will be beneficial, neutral or harmful. An attempt was made

TABLE 3
THE QUANTITATIVE NITROGEN METABOLISM OF *B. ACIDOPHILUS* TYPE I

Mg. per 100 C c.	Day	Control Gelatin Broth	Gelatin Broth	Control Glucose Gelatin	Glucose Gelatin	Control Lactose Gelatin	Lactose Gelatin	Control Milk	Milk
Total nitrogen.....	1	924	924	889	889	889	889	441	441
Protein nitrogen.....		749	777	721	742	721	756	385	378
Nonprotein nitrogen..		175	147	168	147	168	133	56	63
Polypeptid nitrogen..		130.2	95.2	126.7	95.2	128.8	86.8	30.1	39.2
Amino nitrogen.....		39.9	46.9	36.4	47.6	33.6	42.0	20.3	17.1
Ammonia nitrogen...		4.9	4.9	4.9	4.2	5.6	4.2	5.6	6.3
Total nitrogen.....	3	924	924	889	889	889	889	441	441
Protein nitrogen.....		749	770	721	770	721	784	385	378
Nonprotein nitrogen..		175	154	168	119	168	105	56	63
Polypeptid nitrogen..		130.2	109.2	126.7	72.1	128.8	58.8	30.1	35.0
Amino nitrogen.....		39.9	39.9	36.4	42	33.6	41.3	20.3	21.7
Ammonia nitrogen...		4.9	4.9	4.9	4.9	5.6	4.9	5.6	6.3
Total nitrogen.....	6	924	924	889	889	889	889	441	441
Protein nitrogen.....		749	777	721	763	721	784	385	385
Nonprotein nitrogen..		175	147	168	126	168	105	56	56
Polypeptid nitrogen..		130.2	102.2	126.7	78.4	128.8	60.9	30.1	28.7
Amino nitrogen.....		39.9	58.5	36.4	42.0	33.6	39.2	20.3	21.0
Ammonia nitrogen...		4.9	6.3	4.9	5.6	5.6	4.9	5.6	6.3
Total nitrogen.....	10	924	924	889	889	889	889	441	441
Protein nitrogen.....		749	819	721	770	721	784	385	392
Nonprotein nitrogen..		175	105	168	119	168	105	56	49
Polypeptid nitrogen..		130.2	52.5	126.7	72.1	128.8	59.5	30.1	24.5
Amino nitrogen.....		39.9	47.6	36.4	42.7	33.6	41.3	20.3	17.5
Ammonia nitrogen...		4.9	4.9	4.9	4.2	5.6	4.2	5.6	7.0

to throw light on this problem by cultivating the various types in mediums which are representative in type, if not in detail, of those which may be found within the alimentary canal of man.

Mediums containing the usual constituents of plain nutrient gelatin (5%) and some reinforced with glucose and lactose, and milk, were selected as representative. A survey of the fermentation tables shows quite clearly that a varied assortment of utilizable carbohydrates would add little or nothing to the information gleaned from a study of the metabolism of *B. acidophilus* in the plain, lactose and milk mediums.

Tables 3, 4 and 5 set forth in detail the quantitative changes induced in the mediums mentioned above by 3 types of *B. acidophilus*. The

TABLE 4
BACTERIAL METABOLISM OF *B. ACIDOPHILUS*, TYPE II

Mg. per 100 C c.	Day	Control Gelatin Broth	Gelatin Broth	Control Glucose Gelatin	Glucose Gelatin	Control Lactose Gelatin	Lactose Gelatin	Control Milk	Milk
Total nitrogen.....	1	896	896	861	861	861	861	455	455
Protein nitrogen.....		686	679	644	637	651	651	399	406
Nonprotein nitrogen..		210	217	217	224	210	210	56	49
Polypeptid nitrogen..		169.4	176.4	175.7	185.5	168.7	172.9	31.5	23.8
Amino nitrogen.....		35.7	35.7	36.4	32.9	37.1	32.9	18.9	22.4
Ammonia nitrogen...		4.9	4.9	4.9	5.6	4.2	4.2	5.6	2.8
Total nitrogen.....	3	896	896	861	861	861	861	455	455
Protein nitrogen.....		686	735	644	700	651	735	399	406
Nonprotein nitrogen..		210	161	217	161	210	126	56	49
Polypeptid nitrogen..		169.4	119.7	175.7	116.2	168.7	86.8	31.5	19.6
Amino nitrogen.....		35.7	35.0	36.4	38.5	37.1	35.0	18.9	24.5
Ammonia nitrogen...		4.9	6.3	4.9	6.3	4.2	4.2	5.6	4.9
Total nitrogen.....	6	896	896	861	861	861	861	455	455
Protein nitrogen.....		686	756	644	707	651	714	399	392
Nonprotein nitrogen..		210	140	217	154	210	147	56	63
Polypeptid nitrogen..		169.4	98.7	175.7	107.8	168.7	100.8	31.5	32.9
Amino nitrogen.....		35.7	37.1	36.4	42.0	37.1	42.7	18.9	23.8
Ammonia nitrogen...		4.9	4.2	4.9	4.2	4.2	3.5	5.6	6.3
Total nitrogen.....	8	896	896	861	861	861	861	455	455
Protein nitrogen.....		686	707	644	686	651	721	399	392
Nonprotein nitrogen..		210	189	217	175	210	140	56	63
Polypeptid nitrogen..		169.4	145.6	175.7	131.6	168.7	93.8	31.5	30.8
Amino nitrogen.....		35.7	39.2	36.4	38.5	37.1	42.0	18.9	26.6
Ammonia nitrogen...		4.9	4.2	4.9	4.9	4.2	4.2	5.6	5.6
Total nitrogen.....	10	896	896	861	861	861	861	455	455
Protein nitrogen.....		686	749	644	672	651	672	399	385
Nonprotein nitrogen..		210	147	217	189	210	189	56	70
Polypeptid nitrogen..		169.4	102.2	175.7	141.4	168.7	142.1	31.5	39.2
Amino nitrogen.....		35.7	39.2	36.4	43.4	37.1	42.0	18.9	24.5
Ammonia nitrogen...		4.9	5.6	4.9	4.2	4.2	4.9	5.6	6.3

TABLE 5
BACTERIAL METABOLISM, *B. ACIDOPHILUS*, TYPE III

Mg. per 100 C c.	Day	Control Gelatin Broth	Gelatin Broth	Control Glucose Gelatin	Glucose Gelatin	Control Lactose Gelatin	Lactose Gelatin	Control Milk	Milk
Total nitrogen.....	1	917	917	847	847	847	847	455	455
Protein nitrogen.....		770	770	707	707	700	700	399	413
Nonprotein nitrogen..		147	147	140	140	147	147	56	42
Polypeptid nitrogen..		100.8	100.8	98.0	94.5	94.3	93.6	32.2	18.2
Amino nitrogen.....		39.9	39.2	36.4	39.2	37.1	37.8	18.9	18.2
Ammonia nitrogen...		6.3	7.0	5.6	6.3	5.6	5.6	4.9	5.6
Total nitrogen.....	3	917	917	847	847	847	847	455	455
Protein nitrogen.....		770	784	707	714	700	707	399	406
Nonprotein nitrogen..		147	133	140	133	147	140	56	49
Polypeptid nitrogen..		100.8	86.8	98	87.8	94.3	95.2	32.2	24.5
Amino nitrogen.....		39.9	39.2	36.4	39.2	37.1	39.2	18.9	18.9
Ammonia nitrogen...		6.3	7.0	5.6	6.3	5.6	5.6	4.9	5.6
Total nitrogen.....	6	917	917	847	847	847	847	455	455
Protein nitrogen.....		770	770	707	707	700	693	399	406
Nonprotein nitrogen..		147	147	140	140	147	154	56	49
Polypeptid nitrogen..		100.8	103.6	98	94.5	94.3	110.6	32.2	25.9
Amino nitrogen.....		39.9	37.1	36.4	38.5	37.1	37.8	18.9	16.8
Ammonia nitrogen...		6.3	6.3	5.6	7.0	5.6	5.6	4.9	6.3
Total nitrogen.....	8	917	917	847	847	847	847	455	455
Protein nitrogen.....		770	770	707	714	700	700	399	406
Nonprotein nitrogen..		147	147	140	133	147	147	56	49
Polypeptid nitrogen..		100.8	98.7	98	86.1	94.3	99.4	32.2	21.0
Amino nitrogen.....		39.9	42.0	36.4	41.3	37.1	41.3	18.9	22.4
Ammonia nitrogen...		6.3	6.3	5.6	5.6	5.6	6.3	4.9	5.6
Total nitrogen.....	10	917	917	847	847	847	847	455	455
Protein nitrogen.....		770	770	707	714	700	707	399	399
Nonprotein nitrogen..		147	147	140	133	147	140	56	56
Polypeptid nitrogen..		100.8	96.6	98	82.6	94.3	92.6	32.2	29.4
Amino nitrogen.....		39.9	44.8	36.4	45.5	37.1	39.2	18.9	22.4
Ammonia nitrogen...		6.3	5.6	5.6	4.9	5.6	4.2	4.9	4.2

methods employed have been described previously.²¹ The results are expressed in milligrams of nitrogen per 100 c. c. of culture medium.

The results are clear-cut and summarizable in a word: The nitrogenous changes induced by the members of the acidophilus group consist chiefly of a slight increase in the "protein nitrogen" fraction of the medium, with a corresponding diminution in the "polypeptid" fraction. This means in essence that the increase in the protein nitrogen fraction is a measure of the actual body protein of the microbes which build up their substance principally at the expense of the simpler amino acid complexes of the mediums. Neither the ammonia nitrogen fraction nor the amino nitrogen fraction undergoes any material change. In other words, the intracellular utilization of protein derivatives even in mediums free from added carbohydrate is minimal. The quantitative nitrogenous changes, indeed, are almost within the limits of precision of the methods employed.

The results are quite in accord with the qualitative observation that members of the aciduric group of bacteria are of the obligately fermentative type. The extremely slight nitrogenous metabolism of these organisms, the absence of aromatic amines, and of other nitrogenous decomposition of the amino acids with cyclic radicals among their metabolic products, would seem to confirm their claim to suitability for intestinal implantation. It is no accidental coincidence to find acidophili occurring naturally in the human intestinal tract. Members of the aciduric group possess the requisite chemical inertness with reference to their nitrogenous metabolism to be innocuous to their host, and they possess the additional prime requisite—pointed out long ago^{8, 18, 22} but apparently overlooked by most subsequent observers—of thriving in the human alimentary canal.

There can be no doubt that a considerable variety of bacteria possess this requisite benign nitrogenous metabolism, which would suggest their fitness for intestinal implantation; but few of these are endowed with the ability of growing in the intestinal tract, however.²³ It might be remarked in passing that Nature has shown the pattern and selected the microbes that are most adaptable to intestinal conditions. Mankind is ever slow to learn from Nature.

This perusal of the fermentation reactions, the generation of acids in fermentation mediums, and the nitrogenous metabolism of the 3

²¹ Kendall: *Jour. Infect. Dis.*, 1922, 30, p. 211.

²² Rotch and Kendall: *Amer. Jour. Dis. Child.*, 1911, 2, p. 30.

²³ Kendall: *Amer. Jour. Med. Sc.*, 1918, 156, p. 157.

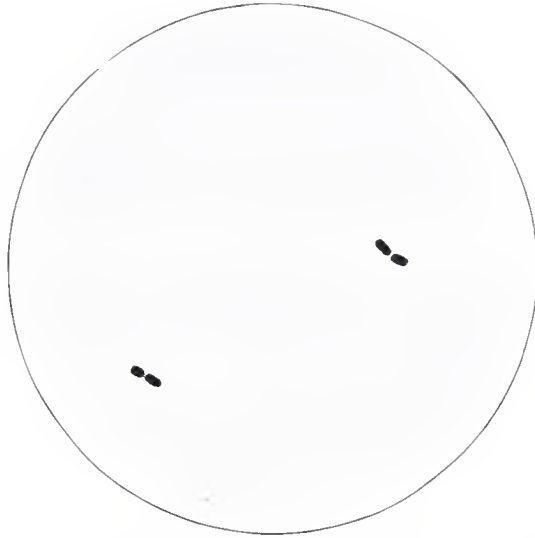


Fig. 1.—*B. acidophilus*, Type 1; plain broth culture; $\times 1200$.



Fig. 2.—*B. acidophilus*, types 1 and 2; lactose broth culture; $\times 1200$.

types of acidophili has shown a group of microbes so similar in chemistry that they might be regarded as essentially identical. What, then, is the basis for considering them as of 3 types?

Morphologically the organisms do exhibit considerable variation in size and shape. These variations are of two kinds: Individual differences in appearance in different mediums, and type differences in size and general microscopic appearance. Reference to figs. 1-9 inclusive will indicate the general nature of the morphologic characteristics better than any description. These are of more than academic interest in that



Fig. 3.—*B. acidophilus*, types 1 and 2; milk culture; $\times 1200$.

a microscopic study of the Gram stained preparations from culture or feces will reveal a considerable variety of forms which, if not clearly understood, will lead to false conclusions about the identity of the bacteria.

In general, the plain broth cultures of members of the acidophilus group (fig. 1) reveal single organisms, except type 2, which is distinctly filamentous (fig. 4). In lactose mediums the growth is more luxuriant and somewhat more beaded. Figure 6 (type 2, lactose culture) illustrates a not uncommon curled group of microbes which may be a degenerative phenomenon, although the culture is otherwise luxuriant. As gas



Fig. 4.—*B. acidophilus*, type 2; plain broth culture; \times 1200.



Fig. 5.—*B. acidophilus*, type 2; glucose agar culture; \times 1200.



Fig. 6.—*B. acidophilus*, type 2; lactose broth culture; $\times 1200$.

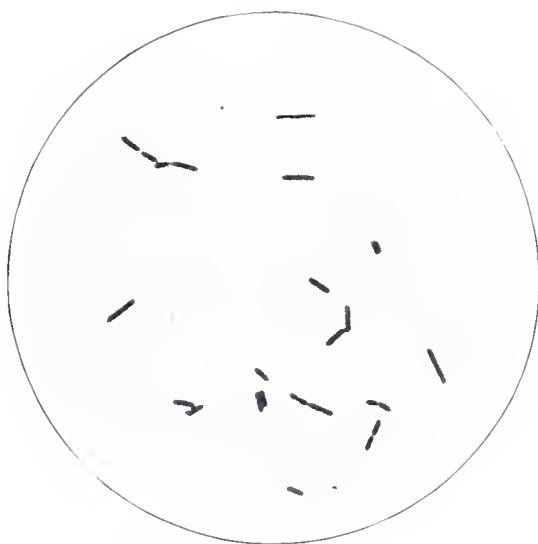


Fig. 7.—*B. acidophilus*, type 3; plain broth culture; $\times 1200$.

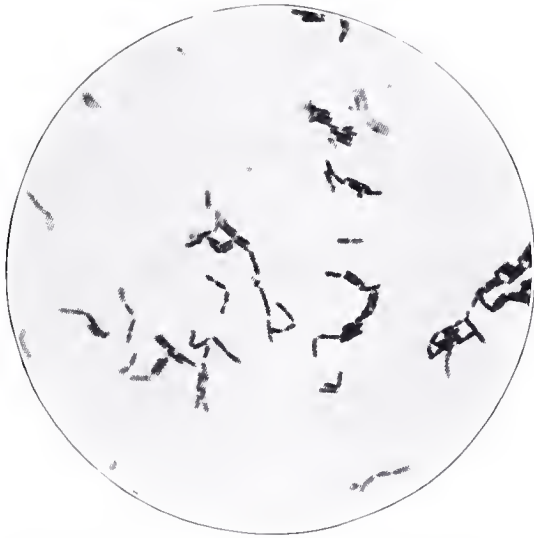


Fig. 8.—*B. acidophilus*; type 3; lactose broth culture; $\times 1200$.



Fig. 9.—*B. acidophilus*, type 3; milk culture; $\times 1200$.

bacilli, and less commonly other bacteria, manifest this same type of growth at times, it cannot be regarded as distinctive. In milk, the members of types 1 and 2 (illustrated by fig. 3) are quite filamentous, reminiscent of the plain broth culture of type 2 (fig. 4), and suggestive of incomplete fission of the individual bacilli. Irregular staining is almost always an accompaniment of this luxuriant, filamentous growth.

The clubbed ends, irregular outline, and bifurcation shown in figure 5 (type 2, glucose agar slant), suggest *B. bifidus* in appearance but not in size.⁷ Type 3 (figs. 7-9) illustrate a variant of the Aciduric group that does not seem to express itself in filamentous form. It is a relatively luxuriant, but rather slow growing type, quantitatively unlike types 1 and 2.

The three types indeed are somewhat dissimilar in their acid production in milk cultures, as is shown in the tables. Whether these differences are maintained over long periods of cultivation or whether they represent somewhat transient quantitative variations can not be stated with finality at this time.

An attempt to immunize rabbits with the three morphologic types of acidophili and thus obtain some serums specific for the chemical architecture of each type respectively was unsuccessful.

SUMMARY AND CONCLUSION

The results of this study have corroborated ideas of the qualitative fermentative reactions of the aciduric group so far as available evidence shows, and, more important, have revealed the singularly negative character of their nitrogenous metabolism, characteristic of the obligately fermentative types of naturally occurring intestinal microbes. This is significant in light of the relation between a carbohydrate-rich, protein-poor diet, which encourages the aciduric group in the human intestinal tract, and the nature of the regimen which must be pursued to induce successful growths of acidophili in an implantation therapy.

The true secret of acidophilic and other lactic acid therapy is not to emphasize the administration of the microbe—but to regard the proliferation of the microbe in the intestinal tract merely as an indication of the establishment of a correct and successful regimen. From the point of view of the host, the dietary condition in the alimentary canal rather than the microbe is the goal to attain.

OPTIMUM AND LIMITING HYDROGEN-ION CONCENTRATIONS FOR *B. BOTULINUS* AND
QUANTITATIVE ESTIMATION
OF ITS GROWTH. XVI

CARRIE CASTLE DOZIER

*From the George Williams Hooper Foundation for Medical Research, University of California
Medical School, San Francisco*

*Aided by grants from the National Cannery Association, the Cannery League of California
and the California Olive Association*

What are the optimum nutritional substances for *B. botulinus*? This was one of the first inquiries to demand attention. The mapping out of a line of approach was complicated by total lack of knowledge regarding the minimum growth requirements of the organism. Van Ermengem¹ failed to secure growth of this anaerobe in synthetic mediums, a subject on which subsequent work with other organisms undoubtedly throws some light. It seems not improbable that the source of structural carbon is of considerable importance in synthetic mediums. The results of Bierema² are of interest; he concluded that micro-organisms can utilize most nitrogen compounds when an appropriate carbohydrate is present. It will be remembered that Proskauer and Beck³ were unable to cultivate the tubercle bacillus on a synthetic medium without glycerol, and Doryland⁴ found that the depressing effect of dextrose on the accumulation of ammonia in a casein solution by 6 "ammonifying" bacteria "is due largely to dextrose serving as a source of energy, thereby allowing the organisms to consume some of the ammonia liberated by them from casein." Long⁵ grew tubercle bacilli readily on certain amino acids as a sole source of nitrogen, but concluded: "As regards the utilization of the carbon in amino acids for structural purposes, it may be said that the tubercle bacilli are unable to synthesize all the carbonaceous portion of their body substance from the material left after the abstraction of nitrogen." Since *B. botulinus*, so far as is known at present, requires more complex nitrogenous food than *B. tuberculosis*, it seems not unlikely that its requirements in the way of carbon for structural and perhaps also for energy

Received for publication, Jan. 30, 1924.

¹ Handbuch f. path. Mikroorganismen, 1913, 4, p. 909.

² Centralbl. f. Bakteriologie, II, 1909, 23, p. 672.

³ Ztschr. f. Hyg. u. Infektionskr., 1894, 18, p. 128.

⁴ Bull. 166. N. D. Agric. Exper. Station, 1916.

⁵ Am. Rev. Tuberc., 1921, 5, p. 725.

needs may be more exacting as well. This hypothesis is supported by the recent work of Braun and Cahn-Bronner.⁶ They found that there must be provided an especially adapted food for energy needs, as well as a complex organic nitrogen source, in order that bacteria may grow and reproduce without atmospheric oxygen.

Theobald Smith and his collaborators⁷ realized this need of anaerobes for suitable food: "The essential problem in the study of anaerobes is not so much the exclusion of oxygen as the supply of special kinds of assimilable foods." While glucose is attacked readily by *B. botulinus*, it is conceivable that a more acceptable form of carbon may exist. The facility with which it grows on vegetable mediums suggests this. Vegetables in general furnish a comparatively large amount of stored carbonaceous food material. Lipman,⁸ using soil organisms, found starch depressed the accumulation of ammonia more than did glucose. His results were interpreted by Doryland⁴ to mean that the carbohydrate enabled the bacteria to use the ammonia, in which case it may be concluded that the starch furnished some substance more acceptable to the organisms than glucose. Waksman⁹ found actinomycetes used starch more readily than glucose. The growth-stimulating property of Vedder's¹⁰ starch agar for gonococci, and Avery's¹¹ sodium oleate agar for *B. influenzae* are examples of more acceptable forms of carbonaceous food for certain organisms than the sugars commonly offered to bacteria. *B. botulinus* is primarily a soil organism, and while glucose under laboratory conditions is the most acceptable form of carbohydrate known, under natural conditions it probably furnishes an insignificant portion of the organism's food.

Early attempts to secure growth of *B. botulinus* in mediums in which single amino acids furnished the sole source of nitrogen met with questionable success. While this problem is being investigated further by other workers in this laboratory, it was thought after a few preliminary experiments, the progress of the investigation of the limiting and optimum hydrogen-ion requirements, the inhibitive effect of sugars and sodium chloride on the viability and growth of vegetative and spore forms, and the resistance of spores to disinfectants, would be served best by a critical examination of the growth-promoting properties for

⁶ Biochem. Ztschr., 1922, 131, p. 272.

⁷ Jour. Med. Res., 1905-06, 14, p. 193.

⁸ N. J., Ann. Rep. of the Agric. Exper. Sta., 1909, p. 117.

⁹ Jour. Bacteriol., 1919, 4, p. 307.

¹⁰ Jour. Infec. Dis., 1915, 16, p. 385.

¹¹ Jour. Am. Med. Assn., 1918, 71, p. 2050.

B. botulinus of some of the usual laboratory mediums and adjuvants, such as peptones and other growth-stimulating substances, including blood and vitamins.

“BIOLOGIC VALUE”

At an early stage in the work a need was felt for an expressive word or phrase to describe differences in the ability of mediums to support bacterial growth and reproduction. Many observations have suggested the similarity of the fundamental part played by the products of protein hydrolysis in animal and bacterial nutrition. Rubner's¹² calorimetric determinations of the energy exchange during bacterial growth indicated the parallelism of animal and bacterial metabolism. Kendall¹³ has recently reviewed his evidence allowing an extension of the conception of “the protein sparing power of carbohydrates” to bacterial nutrition, and his designation of ammonia, which he considers an index of endogenous bacterial metabolism, as “bacterial urea.” “Biologic value,” a term first used by Thomas¹⁴ in expressing the unequal nutritional value of proteins, has come to be an indispensable part of the phraseology employed by students of animal and human nutrition. The facts just discussed seem to justify its extension to express differences in the nutritional value of different mediums to support bacterial growth and reproduction. In this sense it will be used in subsequent pages.

CHOICE OF A STANDARD MEDIUM

Considerable difficulty was experienced at the outset in consistently securing growth of *B. botulinus* in double strength veal infusion—2% peptone-phosphate buffered medium, which it was planned to use for certain growth determinations. It was soon evident that commercial peptones differ greatly in biologic value for *B. botulinus*, and following a series of comparative trials, Difco peptone was chosen as the brand which could be relied on to support representative growths of the organism. The technic employed in preparing such mediums was that outlined by Schoenholz and Meyer.¹⁵

Effect of Phosphates on the Growth of B. Botulinus.—From a study of experimental results, it was concluded that monobasic and dibasic phosphates in the concentration it was proposed to employ in later work

¹² Arch. f. Hyg., 1906, 57, p. 193.

¹³ Endocrinology and Metabolism, 1922, 3, p. 346.

¹⁴ Arch. f. Anat. u. Physiol., 1909, p. 219.

¹⁵ Jour. Infect. Dis., 1921, 28, p. 384.

(less than a maximum of 0.1 molal) do not inhibit the growth of *B. botulinus*. Furthermore, macroscopic growth determinations, as well as toxin tests on guinea-pigs, suggest that sodium and potassium may be used interchangeably within such range. It was noted too, that the buffer index¹⁶ of 0.1 molal concentrations in standard mediums is such that the P_H is not depressed beyond the optimum zone by the growth of *B. botulinus* for 4 to 10 days, inclusive.

Growth-Stimulating Effect of Blood and Vegetable Extracts.—No attempt was made to study the mode of action of the growth-stimulating power of blood, but results show the nutritional significance of blood in double strength veal infusion—2% peptone for *B. botulinus* to be polyphasic; appreciably lowering the bacteriostatic value of hydrogen-ion concentration and size of inoculum necessary to initiate growth, minimizing the lag period, and carrying the growth curve to a higher maximum.

Following the technic of Thjötta and Avery,¹⁷ tomato essence was prepared and added up to a maximum of 10% to both liquid and solid mediums. It was anticipated that the addition of this clear, yellow essence would sufficiently enrich the usual agar shake mediums (glucose-veal and glucose-liver agar) to stimulate the growth and reproduction of all the viable organisms inoculated. Repeated trials failed to demonstrate any activating influence. The P_H of the tomato essence determined electrometrically was 4.17; that of the double strength veal infusion—2% Difco peptone used in one set of experiments before the addition of the essence, 7.32; after addition, 6.86. It is clear in view of the wide optimum range of hydrogen-ion concentration demonstrated in subsequent paragraphs that increased acidity of the medium is not responsible for the nonaccelerating action. Tomatoes were chosen because of the well-known triple vitamin content.¹⁸

This lack of stimulation is of interest in connection with its variance from Kligler's¹⁹ conclusion, based on work with tissues, however, that "the water soluble substances are apparently the ones essential for bacterial development," and the similar conclusions of Davis²⁰ and Leichtenritt²¹ that the significance of extracts containing vitamins is similar for bacterial and animal life. However, the conclusions of Funk and

¹⁶ Brown: Jour. Bacteriol., 1921, 6, p. 555.

¹⁷ Jour. Exper. Med., 1921, 34, p. 455.

¹⁸ Osborne and Mendel: Jour. Biol. Chem., 1920, 41, p. 451; p. 549. Givens and McCluggage: Ibid., p. 24.

¹⁹ Jour. Exper. Med., 1919, 30, p. 31.

²⁰ Jour. Infect. Dis., 1921, 29, p. 171.

²¹ Monatschr. f. Kinderheilk., 1921-22, 22, p. 375.

Dubin²² and Ayers and Mudge²³ are not in harmony with such a view, and it is of interest to note that more rigid criteria are eliminating the yeast test as a measure of vitamin B.²⁴

Beef Heart Mediums.—Heart muscle, since Funk²⁵ in 1913 observed that "these substances (vitamins) are found in meat, especially abundant in heart muscle," has frequently been demonstrated to have a high biologic value. The heat stability of its growth-promoting properties was first noted by Osborne and Mendel.²⁶ They reported satisfactory growth of white rats on pig heart muscle which had been heated at 90 C. for several hours, in contradistinction to failure of growth on similarly treated skeletal muscle.²⁷ Heart muscle mediums subjected to the ordinary methods of sterilization have been acknowledged many times to possess high biologic value for bacteria. It is worthy of note that Huntoon's²⁸ "hormone medium," whose superior biologic value has been ascribed entirely to its method of manufacture without filtration, is made of heart instead of voluntary muscle. Robertson's²⁹ bullock heart "cooked meat medium," highly recommended by Holman,³⁰ is one the superior value of which is now recognized generally by workers with anaerobes. Lloyd's³¹ choice of various substratums investigated for the primary isolation of gonococci was beef heart infusion. Mueller³² advises that beef heart plus glucose and inorganic salts is a satisfactory medium for the growth of hemolytic streptococci. The only dissenting voice is that of Kligler,¹⁹ who found the stimulating properties of beef heart infusion for bacteria affected adversely by heating for short periods.

It is not feasible to give an extended account of the work on the evaluation of the biologic value of beef heart mediums for *B. botulinus*, but it may be stated that all results attest to its superior nutritive value for this anaerobe. Results of recent tests for the sugar content of heart muscle conducted by Wolf³³ are of interest. He failed to find any glucose in either pig or bullock heart, which is suggestive in view of the previously discussed possibility of a source of carbon more

²² Jour. Biol. Chem., 1921, 48, p. 437.

²³ Jour. Bacteriol., 1922, 7, p. 449.

²⁴ Fleming: Jour. Biol. Chem., 1921, 49, p. 119. Eijkman, van Hoogenhuijze and Derks: Ibid., 1922, 50, p. 311. Fulmer and Nelson: Ibid., 51, p. 77.

²⁵ München. med. Wchnschr., 1913, 60, II, p. 2614.

²⁶ Jour. Biol. Chem., 1918, 34, p. 17.

²⁷ Ibid., 32, 1917, p. 309.

²⁸ Jour. Infect. Dis., 1916, 23, p. 169.

²⁹ Jour. Path. & Bacteriol., 1915-16, 20, p. 327.

³⁰ Jour. Bacteriol., 1919, 4, p. 149.

³¹ Jour. Path. & Bacteriol., 1916-17, 21, p. 113.

³² Jour. Bacteriol., 1922, 7, pp. 309 and 325.

³³ Brit. Jour. Exper. Path., 1922, 3, p. 295.

acceptable to *B. botulinus* than glucose. Heller³⁴ advised a pooling of the beef heart with peptic digest for anaerobes, and after several preliminary tests the routine procedure of combining 1 part of beef heart infusion with 2 parts of peptic digest was adopted. Early observations suggested a slight increase in biologic value for *B. botulinus* when some of the beef heart muscle was included, and experiments to test this observation resulted in confirming it. These results particularly emphasized the value of the muscle tissue in initiating reproduction and growth with small inoculums, and demonstrated in a less marked manner its superiority in reducing the length of the lag period. Scores of comparisons made possible in various ways during the course of later work led to the conclusion that the muscle-containing medium is the more dependable of the two in recovering small numbers (1 or more) of organisms, especially spores. The addition of glucose (0.5%) enriches the medium for *B. botulinus*. Apparently there is a summation of effect of the enriching qualities of the meat particles and the sugar. Wagner, Dozier and Meyer³⁵ have published quantitative results showing a high level of growth of *B. botulinus* and other anaerobes in such a medium. Blood failed to enhance the biologic value of mediums containing beef heart.

QUANTITATIVE MEASUREMENT OF MICRO-AEROPHILIC GROWTHS

Considerable information was secured by macroscopic measurements of growth. Especially was this true after the adoption of petrolatum instead of paraffin oil seals.³⁶ The petrolatum provides a means of measuring gas production, which in conjunction with turbidity, allows a fair basis for the estimation of the amounts of growth in clear mediums. However, even so, the limitations of such a method were soon apparent, and without a method of measuring growth in opaque mediums the scope of the investigation would have been curtailed seriously.

Vaccine standardization processes were investigated. The several microscopic, volumetric, gravimetric and nephelometric procedures examined will be briefly discussed seriatim.

The Wright method³⁷ failed to give comparable results. This is in accord with the results of Glynn, Powell, Rees and Cox.³⁸ They found there might easily be an error as great as 54% by this method.

³⁴ Jour. Bacteriol., 1921, 6, p. 445.

³⁵ Jour. Infect. Dis., 1924, 34, p. 63.

³⁶ Hall: Jour. Bacteriol., 1921, 6, p. 1. Olitsky and Gates: Jour. Exper. Med., 1921, 33, p. 51.

³⁷ Lancet, 1902, II, p. 11.

³⁸ Jour. Path. & Bacteriol., 1913-14, 18, p. 379.

The direct count, using a Helber chamber,³⁹ was expensive of time and eyesight, but duplicate counts agreed closely, which is in accord with Wilson's⁴⁰ conclusions after a recent critical study of the method.

The Hopkin's tube method⁴¹ measured precipitates and solids as well as bacteria.

The gravimetric method of Wilson and Dickson⁴² may be criticized for the same defect.

The nephelometric method was used successfully by Dunham⁴³ for vaccines and by Dernby and Avery⁴⁴ for measuring growth of pneumococci grown within a hydrogen-ion concentration range of P_H 6 to 8, in which precipitates did not interfere with readings. Measurements are difficult to make by this method unless the base of the standard suspension is the same medium as that of the culture to be compared, and dark colored and opaque mediums are therefore prohibited. *B. botulinus* is a strongly autolytic organism, and because of this tendency a new standard had to be prepared every day. Under optimum conditions, accurate results are to be had by this method, but a desire to use wide ranges of P_H and various opaque mediums led to its abandonment.

The disappearing loop of Gates⁴⁵ offered possibilities, but was rejected for the same reasons as the nephelometric method.

Dilution Methods.—Agar Shakes: Believing that viable organisms should form the basis of counts, trials with the well-known expedient of dilutions in agar shakes were made. The tubes were stratified with petrolatum after seeding to provide anaerobic conditions for the organisms in the top of the agar. The procedure was moderately successful, giving comparable results, but the substratums of glucose-veal and glucose-liver agar used were not sufficiently sensitive to recover all the viable organisms. Colonies would not be visible in opaque mediums.

Liquid Mediums: Dilutions in liquid mediums seemed to offer some advantages, and efforts were directed toward finding the medium which would initiate growth with the smallest number of viable organisms present. Peptic digest-beef heart mince—0.5% glucose was most efficient, and it may be stated that semiquantitative results are possible by such a method.

Plate Counts: Accumulation of much quantitative aerobic bacteriologic data has been made possible by plate counts, but extension of this accepted measurement of growth to the anaerobic field has not been feasible for several reasons. Lack of a medium of high biologic value for numerous species of anaerobes, lack of knowledge of specific colony form, and lack of practical methods of securing anaerobiosis on a comparatively large scale have all contributed to a slow accumulation of knowledge of the various phases of growth of anaerobes. The combination of peptic digest and beef heart infusion which has previously been described as a dependable medium of high biologic value for *B. botulinus* offered a means of approaching the problem of adapting the plating method to anaerobic conditions.

Zeissler⁴⁶ emphasized the specificity of colony form of anaerobes on glucose-human blood agar plates, and later recommended⁴⁷ the substitution of the more

³⁹ Glynn and Cox: *Ibid.*, 1911, 15, p. 360.

⁴⁰ *Jour. Bacteriol.*, 1922, 7, p. 405.

⁴¹ *Jour. Am. Med. Assn.*, 1913, 60, p. 1615.

⁴² *Jour. Hyg.*, 1912, 12, p. 49.

⁴³ *Jour. Immunol.*, 1920, 5, p. 337.

⁴⁴ *Jour. Exper. Med.*, 1918, 28, p. 345.

⁴⁵ *Ibid.*, 1921, 31, p. 105.

⁴⁶ *Ztschr. f. Hyg. u. Infektionskr.*, 1918, 86, p. 52.

easily available horse or sheep blood for routine use. He highly recommends⁴⁸ the use of blood-agar plates for the cultivation, recognition, isolation, and testing of the purity of anaerobes. The results of preliminary tests were in general in agreement with these conclusions, and the medium and technic chosen as entirely suitable for the plate cultivation of *B. botulinus* and other anaerobes is described in the following:

Medium for Blood Plates.—Two parts of peptic digest, 1 part of beef heart infusion, 0.5% glucose, and 1.5% agar, P_H 7.0-7.2 was prepared in 25 liter quantities, bottled in 95 and 190 c.c. amounts, sterilized in flowing steam for 20 minutes on 5 successive days, incubated at 37 C. for 48 hours and stored for use as needed.

Sheep blood was taken aseptically from the jugular vein, defibrinated by shaking with glass beads, and kept in the refrigerator for use as needed. Rabbit blood and horse blood were tested and found to have no advantage over sheep blood. To guard against any possible bactericidal action of fresh blood, it was never used before refrigeration for 12-18 hours, nor was it ever used after two weeks in the icebox.

Technic of Pouring Plates and Incubation in the Improved Anaerobe Jars.—The agar was thoroughly melted in a boiling bath, cooled slightly and placed in a 50 C. water bath for at least 30 minutes. The culture to be tested was well agitated and a sample removed. One c.c. was diluted in 9 c.c. of peptic digest-beef heart infusion—0.5% glucose medium (identical with the medium used for the blood plates except that no agar had been added), and successive dilutions made in the same fluid. A separate sterile pipet was used for each transfer, a precautionary measure which has since been insisted on by Wilson.⁴⁰ An effort was made to plate from dilutions containing approximately 200 viable organisms per c.c. Duplicate plates were made from 3 dilutions usually—sometimes more—depending on the probable accuracy of macroscopic estimation of the number of viable organisms present. And it may be stated that this means of estimating growth was often not reliable. Inoculums of 1 c.c. amounts from the appropriate dilution tubes were made in standard size Petri dishes (100 mm. in diameter) with porous earthenware tops (Arthur H. Thomas catalog No. 25606), using the same pipet in removing duplicate samples, but separate pipets for the removal of samples from successive dilution tubes.

From 7-10% of blood, warmed to 37 C., was added to the nutrient agar, thoroughly shaken, and the whole, in about 15 c.c. amounts, poured into Petri dishes containing the samples. The plates should not have too thick a layer of agar or the colonies will be obscured by disruption of the agar by gas. Neither should the amount be too small or the medium will solidify before the mass of medium and inoculum can be rendered homogenous by gentle rotation. No hard and fast rule can be given because the consistency of the agar will govern to some extent the optimum depth, a softer agar (within the range which it is impossible to govern in making different lots of medium according to the same recipe) allowing the use of a deeper layer. Practice alone will enable the worker to judge this amount with accuracy. Wilson⁴⁰ states that uniform mixing in such plate preparations is difficult to obtain, but no such conclusion could be drawn by the writer. However, a rotating motion was superior to the tipping one usually recommended. The plates were allowed to cool before being placed

⁴⁷ Deutsch. med. Wchnschr., 1918, 44, II, p. 942.

⁴⁸ Kraus and Uhlenhuth in Handbuch der Mikrobiologischen Technik, 1923, 2, p. 961.

in the improved anaerobe jars,⁴⁹ from which the oxygen was then removed. Incubation at 37 C. for 48 hours for cultures containing mainly vegetative forms and 5 days for those seeded with spores was practiced. Comparative tests showed that incubation at 30 C. offered no advantage over incubation at 37 C. Photographs of plates showing typical *B. botulinus* colonies have been published.³⁴

LIMITING AND OPTIMUM HYDROGEN-ION CONCENTRATIONS IN STANDARD MEDIUMS

The relationship existing between acidity and bacterial growth was noted by Pasteur, but present-day conceptions of potential and true acidity—their control by the use of buffers; their measure by titration, by electrolysis, and by the use of indicators; their various applications to bacterial life, death, growth and reproduction, rest on such epoch-making work as that of Arrhenius, Sørensen, Henderson, Clark and Lubs. Arrhenius⁵⁰ conceived the theory of electrolytic dissociation and related it to acidity. Sørensen⁵¹ simplified, perfected and originated tools to measure hydrogen-ion concentration, and Clark and Lubs⁵² further adapted them to bacteriologic technic, while Henderson's⁵³ study of fundamental biochemical acid-base equilibria led him to suggest the use of "natural" phosphates as a means of controlling hydrogen-ion concentration in bacteriologic culture mediums.

Foster,⁵⁴ in 1921, summarized the results then available on limiting and optimum hydrogen-ion concentrations for various species of bacteria. The only anaerobic data included in the summary are those of Wolf and Harris,⁵⁵ who had macroscopically determined the mean value of the "critical Ph" (acid) for *B. perfringens* and *B. sporogenes* to be 4.82 and 4.94, respectively, in 2% glucose-tryptic digest. Slight variations on either side were specific for the acids studied.

Dernby and Blanc⁵⁶ published a composite anaerobic growth curve from data macroscopically derived from 18-hour cultures of *Clostridia sporogenes* A, *sporogenes* O, *canadiense*, *histolyticum*, *putrificum* and *perfringens* in sugar-free autolyzed veal broth. They found the optimum reaction range to be P_H 6.5 to 7.5 and the limiting range P_H 5 to 9, with a mean optimum of P_H 7. Their work will be referred

⁴⁹ Richardson and Dozier: *Jour. Infect. Dis.*, 1922, 31, p. 617.

⁵⁰ Untersuchungen über die galvanische Leitfähigkeit der Elektrolyte, translated by Anna Hamburger, 1907.

⁵¹ Enzymestudien II, *Biochem. Ztschr.*, 1909, 21, p. 131.

⁵² *Jour. Bacteriol.*, 1917, 2, pp. 1, 109 and 191.

⁵³ *Jour. Med. Res.*, 1907, 11, p. 15.

⁵⁴ *Jour. Bacteriol.*, 1921, 6, p. 161.

⁵⁵ *Biochem. Jour.*, 1917, 11, p. 213.

⁵⁶ *Jour. Bacteriol.*, 1921, 6, p. 419.

to further in subsequent paragraphs. Kahn⁵⁷ has stated that he found the optimum P_H for all spore-bearing anaerobes to be 7.2.

Data regarding optimum and limiting reactions for *B. botulinus* are vague and somewhat conflicting. The British Medical Research Committee found⁵⁸ "The organism will not grow in media the reaction of which is acid," while Thom, Edmondson and Giltner's conclusion⁵⁹ is, "*B. botulinus* grows best in slightly alkaline media." The abstract of Itano, Neill and Garvey's⁶⁰ work, for which confirming details have not been published, gives the highly improbable P_H of 10 as the optimum for *B. botulinus*, with limiting concentrations of hydrogen-ion represented by P_H of 5 and 11. Bengtson⁶¹ found an initial reaction of P_H 9 favorable for the production of strong toxin, but this was in a medium with little buffer and 2% of glucose; hence the P_H was undoubtedly soon lowered by acid growth products.

The close relationship of hydrogen-ion concentration to other variables in culture mediums was recognized by Lazarus⁶² in 1908, who wrote:

Les conditions réalisées dans ces expériences permettent d'affirmer qu'un microbe se trouvant en présence d'une quantité déterminée d'ions H ou OH peut se développer ou rester en vie latente, selon la nature des matières nutritives présentes.

Wolf and Harris⁶³ state, "Each organism would appear to have a definite hydrogen-ion concentration in a given medium at which development is impossible." Clark⁶⁴ in his splendid résumé of the whole subject of acidity warns:

The . . . consequent grouping of phenomena about the activity of the hydrogen ion is unfortunate when it confers undue weight upon a subordinate aspect of a problem or when it tends to obscure possibilities of broader generalization.

That he recognized its fundamental significance in bacterial nutrition is evident too, for he also wrote:⁵³

If the membrane is to any degree a mosaic structure with interfaces as sensitive to "reaction" as are those of certain emulsions, then the hydrogen ion concentration of the media must be taken into consideration when dealing with the penetration of food or poison.

⁵⁷ Jour. Med. Res., 1922, 43, p. 155.

⁵⁸ Special Report Series, 39, 1919.

⁵⁹ Jour. Am. Med. Assn., 1919, 73, p. 907.

⁶⁰ Abstr. of Bacteriol., 1920, 4, p. 3.

⁶¹ Am. Jour. Pub. Health, 1921, 11, p. 352.

⁶² Compt. rend., 1908, 65, p. 730.

⁶³ Jour. Path. and Bacteriol., 1916-1917, 21, p. 386.

⁶⁴ The Determination of Hydrogen Ions, 1920, p. 9.

More definite are the statements of Cole and Lloyd, Jones and Foster. Cole and Lloyd's ⁶⁵ results show that "the importance of the reaction is affected to a remarkable degree by the composition of the medium." They found that a medium of high nutrient level broadened the optimum range for gonococci from P_H 7.5-7.8 to 6.7-9.5, or, in other words, compensated for a 10-fold increase in hydrogen ions, and nearly a 100-fold increase in OH ions. Jones ⁶⁶ found "2% whole blood renders the medium of P_H 7.0 even superior to glucose broth of P_H 7.6 in stimulating growth" (pneumococci), and Foster ⁵⁴ concluded, "A marked increase in tolerance for acid is shown by streptococci in the presence of horse serum." Recent studies on the cultivation of gonococci by Torrey and Buckell ⁶⁷ confirm in a general way these conclusions.

A difference in the biologic value of the mediums undoubtedly may account for many of the conflicting limiting ranges of hydrogen-ion concentration brought out by Foster's tabulations. This close relationship of biologic value of the medium and hydrogen-ion concentration has been shown previously for *B. botulinus*, and emphasizes the necessity of stabilizing all other environmental factors when testing for effects of hydrogen-ion concentration on bacterial growth.

Methods of Determining Hydrogen-ion Concentration.—Hydrogen-ion determinations were made by the two generally accepted methods. When measurements of an accuracy of 0.1 P_H or less sufficed, indicators and appropriate buffer solutions of Clark and Lubs ⁶⁴ were used. Whenever greater accuracy than 0.1 P_H was desired, or when dealing with very dark or turbid solutions, hydrogen electrode measurements were made. As a matter of quick convenience all hydrogen electrode measurements are recorded in at least two decimal places, while those determined by means of indicators carry but one decimal place.

SPORE AND VEGETATIVE INOCULUMS

The life cycle of *B. botulinus* may be characterized by two distinct phases, the vegetative and the spore. The latter is physiologically recognizable by its comparatively much greater resistance to unfavorable environmental factors, such as heat, disinfectants and dehydration. During the course of the work unsuccessful efforts were made to secure by 8 to 12-hour transfers in standard medium and also in the same substratum plus 1% glucose, a culture containing vegetative forms only.

⁶⁵ Jour. Path. & Bacteriol., 1916-1917, 21, p. 267.

⁶⁶ Jour. Infect. Dis., 1914, 15, p. 357.

⁶⁷ Ibid., 1922, 31, p. 125.

The presence of spores was not always microscopically demonstrated, but heating of the cultures for varying lengths of time up to a maximum of 2 hours at 80 C. invariably resulted in growth in 1 c.c. subcultures in petrolatum stratified peptic digest-beef heart mince, but seldom in tubes having smaller inoculums. Stains showed healthy gram-positive organisms with only an occasional gram-negative rod. Table 1 gives results which are typical of numerous trials.

The uniform initiation of growth in tubes planted with 1 c.c. of the suspension heated for 15 min., 30 min., and 1 hour, respectively, together

TABLE 1
HEAT RESISTANCE OF HEALTHY 12-HOUR CULTURE OF *B. BOTULINUS* STRAIN 97, INTENSIVELY CULTIVATED FOR 10 DAYS IN VEAL INFUSION—2% PEPTONE—1% GLUCOSE. NO SPORES FOUND IN STAINS EXAMINED. TRANSPLANTS MADE INTO PEPTIC DIGEST-BEEF HEART MINCE, PETROLATUM STRATIFIED

Amount of Original Culture Inoculated, C c.	Minutes Subjected to 80 C.				
	0	15	30	60	120
	Growth in Days	Growth in Days	Growth in Days	Growth in Days	Growth in Days
1.0.....	1	2	2	2	3
0.1.....	1	5	—	—	—
0.01.....	1	—	—	—	—
0.001.....	1	—	—	—	—
0.0001.....	1	—	—	—	—
0.00001.....	1	—	—	—	—
0.000001.....	1	—	39*
0.0000001.....	1	—	—
0.00000001.....	2	—
0.000000001.....	—	—
0.0000000001.....	—
0.00000000001.....	—

— indicates no growth in 6 months; .., no culture.

* Toxic for guinea-pig. Shake culture o.k. Stain satisfactory.

with almost uniform failure of growth in tubes receiving smaller inoculums, denotes a surprising stability of a few of the organisms. After 15 min. of heating growth occurred in the subculture receiving 0.1 c.c. of the heated suspension, suggesting the presence of more viable organisms after 15 min. of heating than after 30 min. In one of the tubes receiving 0.000,001 c.c. of the suspension heated for 30 min. visible growth after 39 days' incubation bespeaks the chance occurrence therein of at least one viable spore. Slight delay in initiation of growth in the 1 c.c. subculture after 2 hrs. of heating is indicative of only slightly greater injury than was sustained by heating for 1 hr. The conclusion seems justified that only well developed spores remain viable after 1 hour's heating at 80 C.

No boiling temperature tests were made, and therefore the results are not directly comparable with data published by Hall,⁶⁸ whose cultures failed to survive 5 and 10 mins. at 100 C., but it is interesting to note that in one case his "supposedly asporogenous culture" did survive heating for 2 hours at 80 C. With a second strain, however, he failed to secure growth in transplants after 80 C. for 1 hour. Conn⁶⁹ experimentally demonstrated that the less resistant soil spores do not withstand a temperature of 85 C. for 15-30 mins., and he finally used a temperature of 75 C. rather than 80 C. to rid his soil samples of vegetative forms. A second fact which precludes direct comparison of results with those of Hall is the difference in the mediums employed for recovery of the organism. Had the more highly selective peptic digest-beef heart mince instead of the glucose broth been used for recovery of the heated spores, it is reasonable to suspect that his cultures might have shown greater heat resistance.

It is doubtful whether a strict line of demarcation can be drawn between vegetative and spore stages as they naturally exist in culture mediums. Hall has suggested the plausible possibility of nondistended spore forms. It is quite within the realm of probability that the cells may be spores physiologically before they can be morphologically detectable. Whatever may be the physiologic significance of the thickened cell wall, it seems impossible, *a priori*, to attribute to it all, or even the greater portion, of the heat resistance of *B. botulinus* spores. The same must be true of spore resistance to disinfectants and drying. While the outer membrane may not have as high a coefficient of permeability to disinfectant as to nutrient solutions, the very fact that a spore will respond to nutritional stimulation after it has successfully resisted exposure to deleterious solutions suggests that the permeability of the cell wall is retained to an effective extent at least. Otherwise it must be granted that the outer covering is a part of the cell capable of initiating processes of growth and reproduction, which is not a function ever ascribed to it by bacteriologists. The constancy of the form of spores of different species of bacteria suggests the efferent course of spore formation, which is described by Jordan⁷⁰ thus: "An assembling or concentration of the nuclear material seems to precede spore-formation in some cases and constitutes the spore primordium."

⁶⁸ Jour. Infect. Dis., 1922, 30, p. 445.

⁶⁹ Jour. Bacteriol., 1906, 1, p. 187.

⁷⁰ General Bacteriology, 1921, p. 77.

The heating of spore suspensions at 80 C. for 1 hour before inoculation has been adopted as a means of disposing of viable vegetative and transitional forms. It is to be emphasized, however, that "vegetative" inoculums, while predominantly vegetative, probably include some spore and transitional forms; while spore inoculums do not contain viable vegetative forms and hence are more homogeneous. Results with spore inoculums, therefore, are undoubtedly less subject to variability. Furthermore, since it has been demonstrated that the soil is the natural reservoir of the organism,⁷¹ and that in common with other telluric sporulating bacteria, it is undoubtedly resident there in the spore stage, experimental work with spore inoculums more nearly simulates natural conditions. The spores were grown in pea-peptic gelatin and the vegetative inoculums were from vigorous 18-hour cultures in double strength veal infusion—2% peptone mediums, P_H about 7.2, in which they had just previously been intensively cultivated for a few days.

Preparation of Mediums.—Several test lots of standard mediums, ranging in P_H from 4 to 10, were planted with various strains of *B. botulinus*, and satisfactory growths secured in every case. Finally 24 one-liter quantities, each having a different hydrogen-ion concentration, were prepared from the same lot of double strength veal infusion, the same bottle of peptone, the same package of NaCl, and the same solutions of phosphates, HCl and NaOH. The P_H was controlled by 0.5 molal HCl or NaOH, and stabilized with appropriate mixtures of monobasic and dibasic sodium phosphates, as recorded by Schoenholz and Meyer.⁷⁵ Ten c.c. amounts of the mediums were placed in sterile test tubes $\frac{5}{8}$ " x 6", covered with $\frac{1}{2}$ inch of sterile petrolatum and autoclaved for 20 min. at 115 C. Sterilization was effected without loss of medium or saturation of the cotton plugs with petrolatum by preliminary heating and careful release of the autoclave pressure during cooling.

After all the mediums had been prepared, the hydrogen-ion concentrations were checked by hydrogen electrode measurements of the contents of two tubes from each set. An average of duplicate determinations differing not more than 0.04 P_H was taken as the hydrogen-ion concentration of each set. If a variation greater than 0.04 was found, another set of determinations was made. The mediums were stored at room temperature and used for tests as rapidly as possible.

Just prior to inoculation the tubes were heated in a boiling water bath for 5 min. and allowed to cool in tepid water which just reached the petrolatum seal. Each tube was quickly seeded with 0.1 c.c. of inoculum from a sterile 1 c.c. pipet graduated in tenths. If, as frequently happened, the petrolatum had hardened enough to make an uneven seal, or an imperfect one, the tube for the length of the petrolatum was heated slightly over the flame of a Bunsen burner.

Preliminary observations suggested that a limit of 72 hours for vegetative inoculums and 14 days when spores are planted gives the best comparative results. A growth curve plotted from an average of the results of 52 tests with vegetative inoculums, using 37 strains of *B. botulinus*, is shown in chart 1. The optimum range of acidity is from P_H 6.0 to 8.2, with a mean optimum approach-

⁷¹ Meyer and Geiger: Pub. Health Rept., 1921, 36, p. 4.

ing neutrality. The limiting range is from P_H 5. to 9. An average of the results of 23 tests with spore inoculums, using 19 strains, shows that the optimum range of P_H extends from 6.0 to 7.2. A definite lengthening of the lag period begins to be appreciable with comparatively slight increase of hydroxyl over

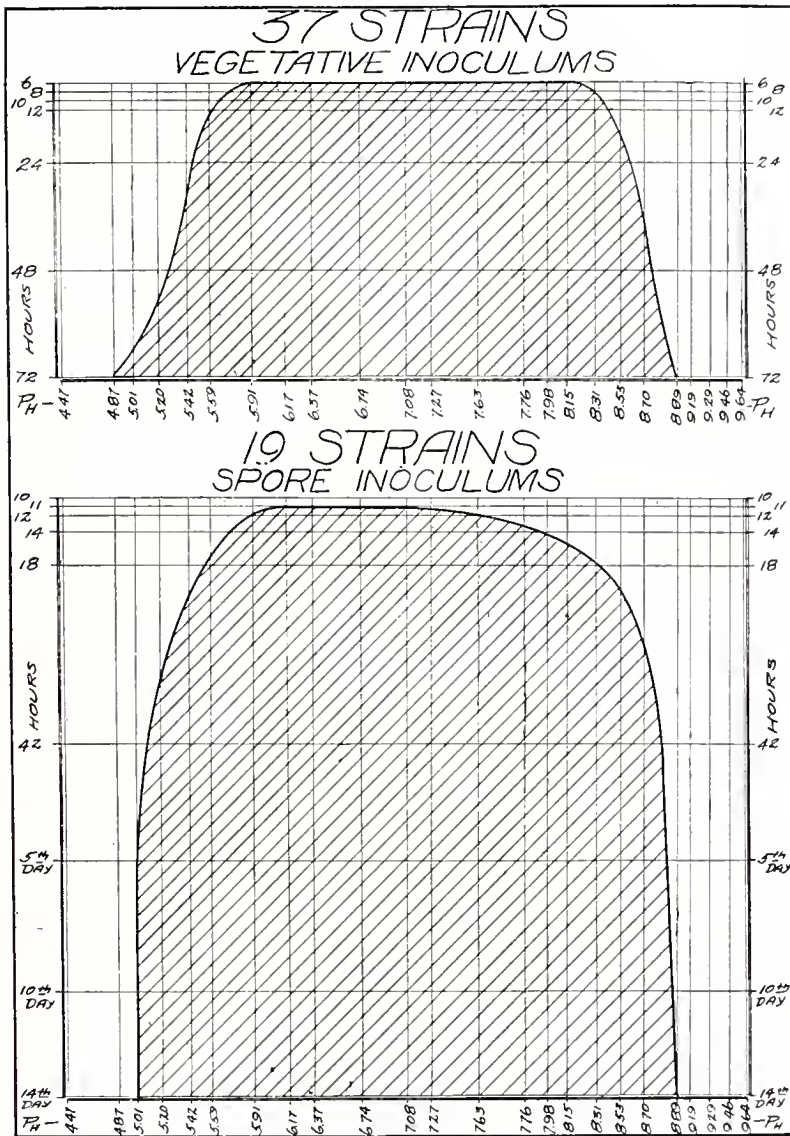


Chart 1.—Composite growth curves of *B. botulinus*.

hydrogen ions. The inhibiting effect is manifested more slowly on the acid side. The optimum hydrogen-ion concentration, in fact, is shown to be approximately P_H 6.6. It seems not unlikely that a slight acidity may be a stimulus to the initiation of metabolic activity. Numerous biologic analogues exist. The stimulating and toxic actions to muscular activity of small and large amounts

of lactic acid are well known. Schoenholz and Meyer¹⁵ cite such diphasic action for bile salts in typhoid cultures. Lehmann⁷² found 0.025 mol HCl had the same effect in hastening the germination of seeds as do light and enzymes, and Eckerson⁷³ concluded the after-ripening period of seeds can be greatly shortened by treating the embryos with dilute acids. Green and Jackson⁷⁴ found that there was an organic acid generated during germination of the seeds of the castor oil plant, while Lehmann and Ottenwalder's⁷⁵ results showed that the optimum concentration for hastening germination is different for the various seeds tested. Further discussion of the stimulating and toxic possibilities of numerous substances is reserved for a later paper dealing with the action of disinfectants on the spores of *B. botulinus*.

QUANTITATIVE ESTIMATIONS OF GROWTHS

Influence of Numbers Inoculated.—Two petrolatum stratified flasks, each with 200 c.c. of sterile standard medium P_H 7.2, were heated in a boiling water bath, cooled and inoculated with 1 c.c. and 10 c.c., respectively, of a 24-day old pea-peptic digest-gelatin culture of strain 38, just previously heated 1 hr. at 80 C. Peptic digest-beef heart mince dilution tubes showed growth in the fifth tube, indicating the presence of 10,000 to 100,000 viable spores per c.c. Plate counts were made from 1 c.c. samples following inoculation, and after 2, 4, 6, 8, 10, 24, 30 and 48 hrs. of growth. The first counts made immediately after inoculation are 857 and 9,725 per c.c., respectively, indicating 173,400 and 195,500 viable organisms per c.c. of seed culture, which is not different from the figure indicated by the dilution count.

At the end of 48 hours' incubation at 37 C., the numbers of viable organisms in the two tubes were approximately equal, but the toxin in the flask receiving the smaller inoculum was only one-tenth as potent for white mice. At the end of 4 days, however, the toxin titers of the two flasks coincided, as shown graphically in chart 2. It should be noted that no determinations were made between the 10th and 24th hours of growth, hence the dotted line portions of the curves between these points are empirically derived. No especial interest attaches to these particular periods of growth in this experiment, and the dotted lines between said points are inserted merely to preserve the continuity of the curves. It is evident that while a 10-fold increase in the size of the inoculum measurably shortens the lag period, toxic and numerical differences in the cultures soon disappear. This is very much like the results found for *Staphylococcus aureus* by Graham-Smith.⁷⁶ It is apparent that the normal curve of growth of *B. botulinus* in its first phases bears a close resemblance to that found for *B. coli*, *B. typhosus* and *B. enteritidis* by Lane-Clayton,⁷⁷ and the curve based on collective aerobic data more closely analyzed by Buchanan.⁷⁸

Effect of Temperature on Growth and Toxin Production by B. botulinus.—Experimental results with 22 strains of *B. botulinus* convinced Orr⁷⁹ that an incubation temperature of 37 C. is the most

⁷² Biochem. Ztschr., 1913, 50, p. 388.

⁷³ Botan. Gaz., 1913, 55, p. 286.

⁷⁴ Proc. Royal Soc. of London, 1905-06, 77, p. 69.

⁷⁵ Ztschr. f. Bot., 1913, 5, p. 337.

⁷⁶ Jour. Hyg., 1920, 19, p. 133.

⁷⁷ Ibid., 1909, 9, p. 239.

⁷⁸ Jour. Infect. Dis., 1918, 23, p. 109.

⁷⁹ Ibid., 1922, 30, p. 118.

favorable for growth, spore production and elaboration of toxin. He points out that this is the temperature which has been preferred generally by bacteriologists in recent years, the only exceptions being Dickson⁸⁰ and Graham and Brueckner.⁸¹ Orr's conclusions have been supported subsequently by Hall.⁶⁸ Kendall,⁸² however, has recently stated that *B. botulinus* should be grown at 30 C. instead of 38 C.

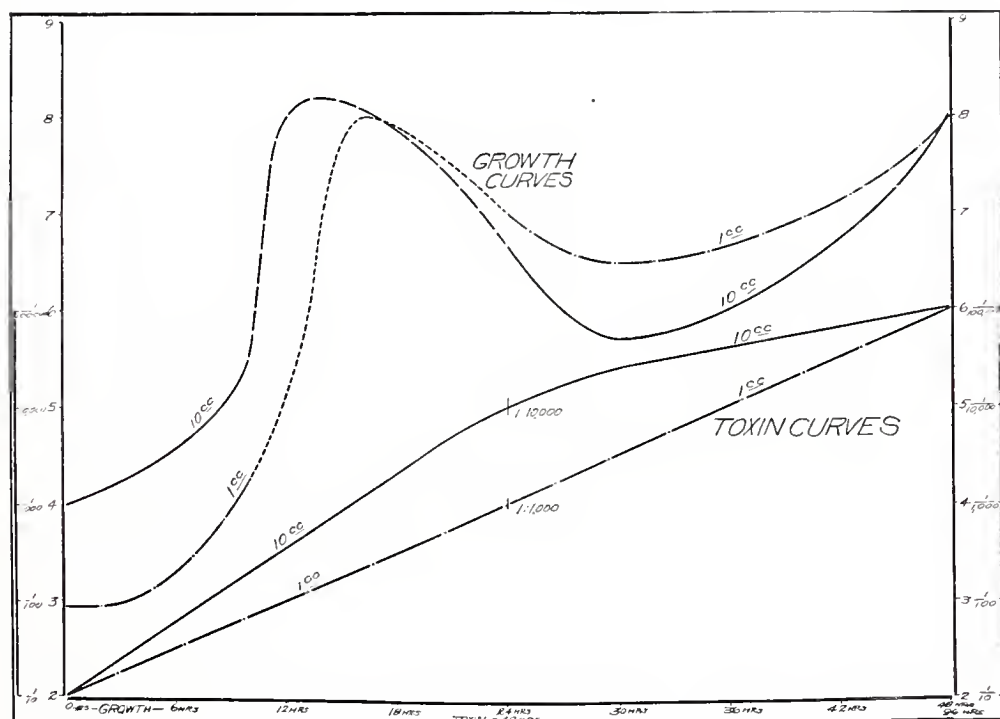


Chart 2.—Influence of numbers inoculated; 1 c.c. and 10 c.c. in 200 c.c.

The toxin titers of strains 38 and 40, grown in standard medium containing 2% glucose, were determined at frequent intervals. One c.c. amounts of progressively increasing dilutions in physiologic salt solution were inoculated subcutaneously into guinea-pigs by means of precision syringes, after the method described by Kolmer.⁸³ One c.c. of sterile saline was used each time to wash out the toxin remaining in the syringes, making the total amount of inoculum in each case 2 c.c. The results of these tests confirm the conclusion that an incubation temperatures of 37 C. is not inimical to the production of a potent toxin. They also are in accord with the conclusions of Bengtson⁶¹ and Dubovsky and Meyer⁸⁴ that the peak of toxicity in cultures incubated at 35-37 C. is reached about the 10th day of growth. Later experimental results with mice,

⁸⁰ Botulism, Monograph of the Rockefeller Institute No. 8, 1918.

⁸¹ Jour. Bacteriol., 1919, 4, p. 1.

⁸² Jour. Infect. Dis., 1923, 32, p. 341.

⁸³ Infection, Immunity and Specific Therapy, 1917, p. 241.

⁸⁴ Jour. Infect. Dis., 1922, 31, p. 501.

however, suggest that a high level of toxicity may be reached after a much shorter incubation period. The relation between the production of toxin and the number of viable organisms present was not studied by Bengtson or Dubovsky and Meyer.

While preliminary tests of growth in solid mediums incubated at 37 C. and at 28 C. had shown a considerable acceleration of growth at the higher temperature, they had not revealed more colonies in tubes receiving equal inoculums. It, therefore, seemed desirable to determine quantitatively the effect of incubating

TABLE 2
TOXIN PRODUCTION IN CULTURES OF *B. BOTULINUS* INCUBATED AT 37 C.

Subcutaneous inoculation into guinea-pigs of supernatant fluid after centrifugation at high speed. Figures show weight of pig in grams and time of death.

Age of Culture in Hrs.	Dilutions											
	Strain 38						Strain 40					
	C e. 1.0	C e. 0.1	C e. 0.01	C e. 0.001	C e. 0.0001	C e. 0.00001	C e. 1.0	C e. 0.1	C e. 0.01	C e. 0.001	C e. 0.0001	C e. 0.00001
6	305	317	—	—	—	—	360	345	—	—	—	—
12	420 13 d.	327	—	—	—	—	410	400	—	—	—	—
24	460 48 hr.	403 8 d.	—	—	—	—	380	325	—	—	—	—
35	372 24 hr.	368 48 hr.	315 12 hr.	—	—	—	295 66 hr.	360	—	—	—	—
48	280 24 hr.	275 48 hr.	260 76 hr.	—	—	—	285 25 hr.	270 48 hr.	265	—	—	—
72	—	305 18 hr.	295 27 hr.	280 5 d.	—	—	265 18 hr.	310 18 hr.	320 48 hr.	290 11 d.	—	—
120	—	—	265 24 hr.	285 53 hr.	320 5 d.	—	—	280 24 hr.	290 48 hr.	260 48 hr.	265	—
8 days	—	—	310 18 hr.	322 41 hr.	290 4 d.	—	—	—	—	300 46 hr.	280	290
11 days	—	—	315 20 hr.	295 48 hr.	320 5 d.	—	—	—	270 24 hr.	305 48 hr.	300 5 d.	—
Filtered			325 20 hr.	295 48 hr.	322 48 hr.	250 5 d.						
16 days	—	—	215 17 hr.	280 47 hr.	365	—	—	—	230 24 hr.	270 48 hr.	270 7 d.	—

similar cultures at room temperature, at 26.5 C. and at 39 C. The latter temperature was selected since experimental data for 37 C. was already at hand, and more particularly, since it was desired to ascertain whether 37 C. is near the upper limit of heat toleration for the growth of *B. botulinus*. Matzschita⁸⁵ reported 45.5 C. as the maximum temperature for such growth. The cultures grown at room temperature were kept in a cupboard in a part of the laboratory where it was thought the minimum amount of fluctuation would occur. The thermograph record for the experimental period shows that the lowest temperature was 12.3 C. and the highest 16.4 C.

⁸⁵ Arch. f. Hyg., 1902, 43, p. 267.

Two hundred c.c. amounts of standard medium, petrolatum stratified, P_H 7.2, inoculated with 18-hour cultures of strains 38 and 40, respectively, were incubated at the three temperatures mentioned. Platings were made on the 18th and 48th hours, and at further intervals of 24 hours. The experimental period for strain 38 lasted 3 days and for strain 40, five days. Strain 40 showed a slightly higher maximum growth at 39 C., but the difference was comparatively slight. The time of attaining the maximum growth was different for each temperature. While the maximum for strain 38 incubated at 39 C. was considerably less than the maxima of sister cultures grown at 26.5 C. and at room temperature, it must

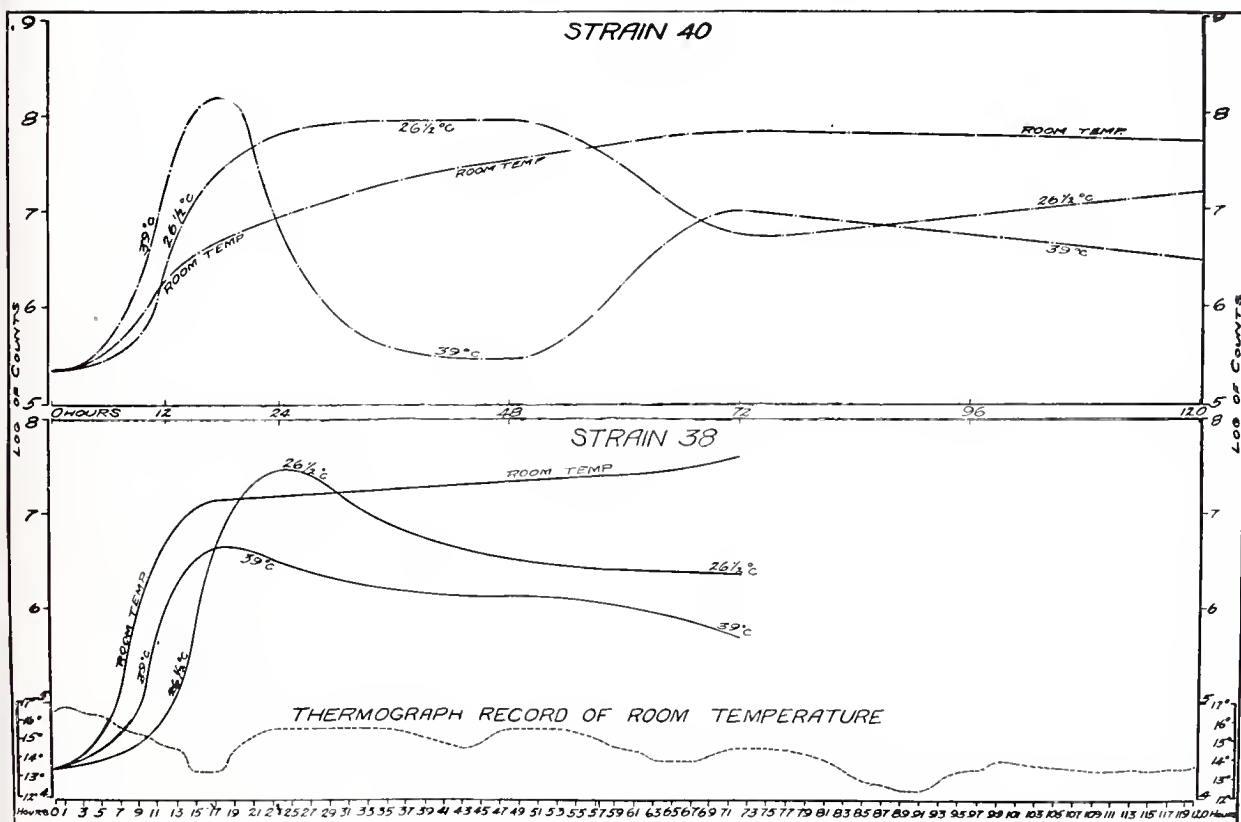


Chart 3.—Effect of temperature on growth in standard mediums.

be kept in mind that the peak probably was reached before the 18th hour, and death had occurred to a considerable extent. This probability is strengthened by the steepness of the room temperature curve and the comparatively narrow plateau of the curve of the 26.5 C. growth, which indicate rapid growth and early death.

In anticipation of a fuller discussion of the probable relationship between growth and toxin production in a subsequent paragraph, it may be stated that these results suggest strongly that toxin production follows closely the death of the organisms and roughly parallels auto-

lysis, or, in other words, toxin production is a function of enzyme action on dead bacterial bodies. Jordan ⁸⁶ showed that gelatinolytic bacterial enzymes are more active at 37 C. than at lower temperatures, "even when the microorganism forming the enzyme grows better at a lower temperature." If this be true for *B. botulinus*, and experimental evidence suggests that it is, toxin production alone cannot be a true index of growth, but is a measure rather of death and disintegration of the bacterial cell.

To summarize, it may be said that these results confirm the conclusions of Orr ⁷⁹ and other investigators so far as they show that *B. botulinus* grows rapidly and produces virulent toxin at the higher incubation temperatures. For maximum numbers of viable organisms, however, incubation at room temperature may be preferable. It seems the most obvious effect of an incubation temperature of 37-39 C. is the acceleration of enzyme activity and consequent cell disintegration, resulting in the early production of toxin.

Influence of Incubation Time on Numbers of Viable Organisms in Standard Mediums With Hydrogen-Ion Concentration Varying from P_H 4.42 to 9.63, Inclusive.—During preliminary studies it was observed that growth in clear mediums, as judged by turbidity, was often not vigorous, although there had been considerable gas formed. Like observations were repeatedly made during work with the nephelometer, and finally when Hopkin's tube measurements were undertaken, a growth volume drop of 50% or more often occurred in standard mediums from one day to the next. Microscopic examination usually revealed few spores, but many gram-negative rods and much debris. As a result of these observations, a series of platings from cultures in standard mediums with hydrogen-ion concentrations varying from P_H 4.42 to 9.63, inclusive, at time intervals of 6, 12, 18, 24, and 48 hrs., was undertaken. Minor changes only occurred in the hydrogen-ion concentrations during the periods of incubation.

A. Six-Hour Growths: As may be noted from chart 3, an optimum P_H approximating neutrality is demonstrated, which confirms the conclusion based on more extensive macroscopic observations. This is in accord also with the findings of Dernby ⁸⁷ and Dernby and Blanc ⁵⁶ for the optimum P_H of the several anaerobes they studied.

⁸⁶ Biological Studies by the pupils of Wm. T. Sedgwick, 1906.

⁸⁷ Ann. de l'Inst. Pasteur, 1921, 35, p. 277.

B. *Twelve-Hour Growths*: The 12-hour curve in chart 3, which is an average of duplicate experiments made with strain 38 on different days, confirms an observation often made during earlier work, that there are small but observable differences in growth in tubes which receive identical treatment so far as possible before and during incubation. While attempts were always made to guard against any differences in the size of inoculums, it is probable that variations well within the limits of experimental error operated to cause these differences in the

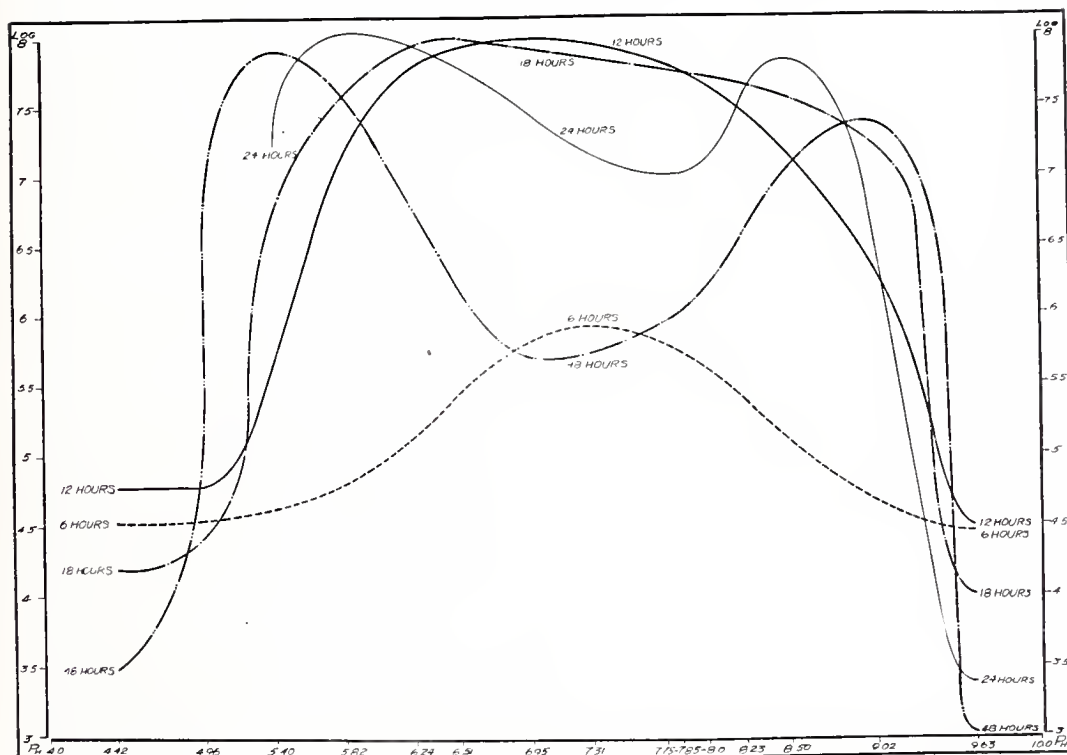


Chart 4.—Growth of *B. botulinus* after 6, 12, 18, 24 and 48 hours of incubation at 37 C.

initiation of growth. A few hours later, however, as has already been shown quantitatively, these differences have disappeared. The 12-hour curve reveals once more an optimum P_H bordering on neutrality.

C. *Eighteen-Hour Growths*: This curve, drawn from an average of four 18-hour growths, shows slightly greater numbers of viable organisms on the acid side of neutrality than on the alkaline side. It seems reasonable to conclude again that the optimum zone is rather broad with a mean near neutrality. These data make it evident that environmental factors causing observable differences in growth at the

end of 12 hrs. have become masked by the end of 18 hrs.; or, in other words, the stabilizing influence of time makes the 18-hour cultures in mediums of the same P_H more nearly alike than are the 12-hour ones. Similar eradication has already been shown for larger differences in size of inoculums by longer time intervals. A practical application of these results is made by continuing to plant from 18-hour cultures when vegetative inoculums are used.

D. *Twenty-Four Hour and Forty-Eight Hour Growths*: The shapes of the curves shown in chart 4 are not surprising in view of former observations. No detailed study has been attempted of the ferments undoubtedly active following the death of the organisms, but subsequent work has demonstrated a gelatinase and a rennin. The not inconsiderable amounts of ammonia formed bespeak an amidase. The shape of the 48-hour curve is almost an exact reversal of that of the 6-hour one, and it seems not unlikely that the hydrogen-ion concentration governing the initiation of growth is indirectly responsible for the initiation of autolysis. No study has been made of the effect per se of the hydrogen-ion concentration on the rate of hydrolysis. It is conceivable that several enzymes are involved analogous to the triple enzyme action found by Dernby for yeasts⁸⁸ and for tissues.⁸⁹

Growth of B. Botulinus, B. Sporogenes and B. Histolyticus in Dernby and Blanc's Medium.—The relationship of P_H to the early growth phases of *B. botulinus* in standard mediums has shown rough correlation with the composite anaerobic growth curve of Dernby and Blanc.⁵⁶ They used an autolyzed veal medium, without added buffer, and on the basis of this data they divide bacteria into two groups: (1) those which have a growth range narrowly limited by the P_H of the medium, and (2) a group showing a wide range of adaptation to hydrogen-ion concentration. In the latter group are included all the anaerobes studied, with the possible exception of *B. tetani*. Results so far recorded indicate that *B. botulinus* belongs to the wide range group, and therefore it seemed of interest to determine quantitatively the range of growth in a medium identical with theirs.

Their technic of medium preparation was followed, with the exception that instead of producing anaerobiosis by use of calcium sulphide powder, this was accomplished by petrolatum stratification before sterilization and heating in a boiling water bath prior to inoculation. Aside

⁸⁸ Biochem. Ztschr., 1917, 81, p. 109.

⁸⁹ Jour. Biol. Chem., 1918, 35, p. 179.

from the addition of another variable by the addition of the powder, there seemed to be less danger of contamination by petrolatum stratification before sterilization. The hydrogen-ion concentrations were determined in duplicate after a few days at room temperature, and as is evident from chart 5, the P_H varied greatly from that of those secured by the foregoing workers. Neither Dernby nor Dernby and Blanc state the amount of inoculums added, although the latter advise that the same

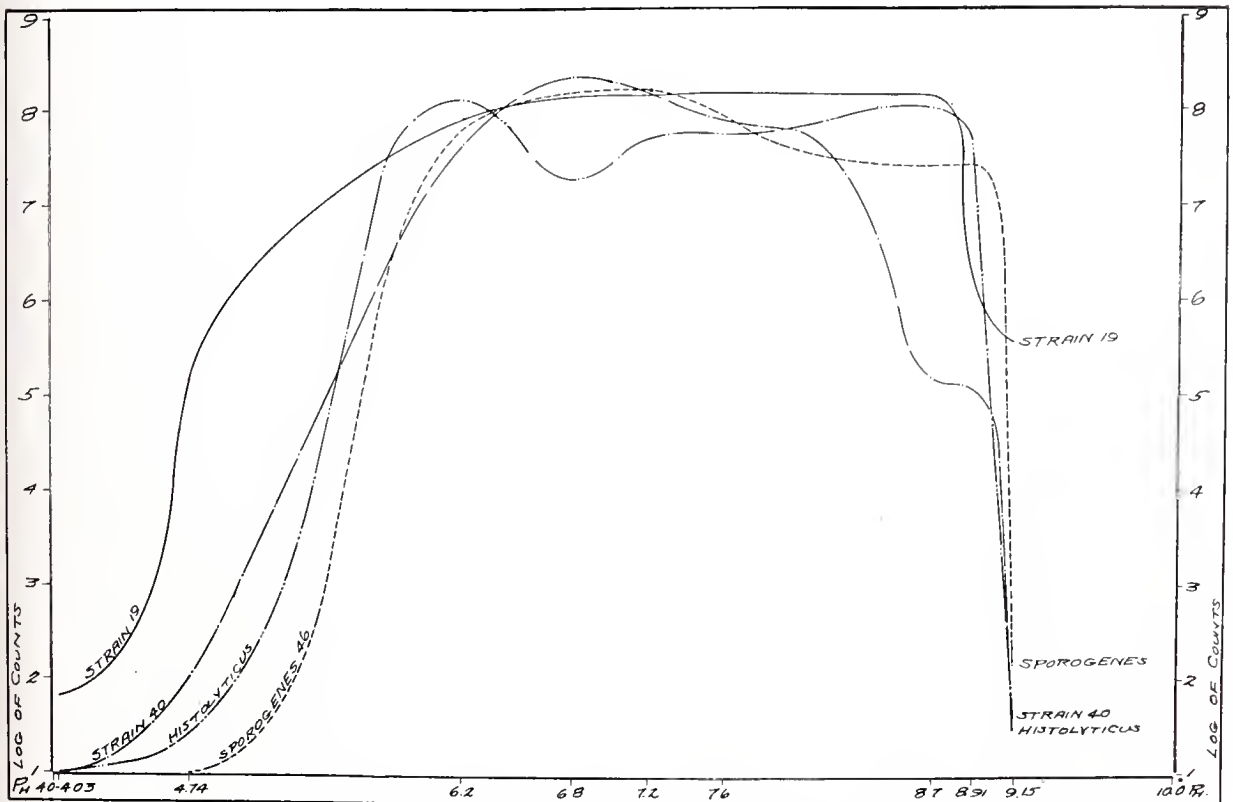


Chart 5.—Growth of *B. botulinus*, *B. sporogenes* and *B. histolyticus* in Dernby and Blanc's medium.

amount of a 24-hour culture was used. My inoculums were from 18-hour cultures and 0.1 c.c. was added to each tube.

Quantitative results showing growth of *B. botulinus* strains 19 and 40, strain 46 of *B. sporogenes* and one strain of *B. histolyticus* after 18 hours incubation at 37 C. are represented graphically in chart 5. The curves differ from Dernby and Blanc's in that a wider optimum range is indicated for 18-hour growths. This range may be stated to be 6.0 to 8.9.

Macroscopic observations of growth were made just prior to plating in the 4 experiments reported. If these results were presented graphically, the plateau of optimum range would be much narrower than quantitative results have shown to be true. Results of 7 additional macroscopic tests substantiate the conclusion that the optimum P_H for the growth of *B. botulinus*, *B. sporogenes* and *B. histolyticus* approximates neutrality. There was close correlation between macroscopic estimations of growth and plate counts with *B. sporogenes* and *B. histolyticus* cultures, but this was not true of results by the two methods of measuring growth in *B. botulinus* cultures. The observation has repeatedly been made that turbidity is not an accurate index of the number of viable botulinus bacilli revealed by plate counts. Extended life cycle studies would undoubtedly throw some light on this complicated question.

Growth of B. Botulinus in Vegetable Mediums.—Prior to the publication in 1918 of Dickson's classic monograph on botulism the only proof which existed of the ability of *B. botulinus* to grow and produce its toxin in vegetable mediums was Gaffky's statement (1) that he had grown the organism in minced beans and secured a potent toxin. Landmann,⁹⁰ in 1904, isolated *B. botulinus* from the remnants of home canned beans which had caused the Darmstadt outbreak. He attributed the growth-promoting properties of these beans for *B. botulinus*, however, to the pork which must have been cooked with them. Dickson cites this as the only record in European literature of the production of true botulism from a foodstuff not of animal origin. Peck,⁹¹ in 1910, incriminated home-canned pears as the causative food responsible for the death of 11 persons. Twelve cases with one death were reported by Wilbur and Ophüls⁹² in 1914, following the eating of home-canned string beans. Curfman, in 1918,⁹³ reported 7 cases with 5 deaths, due to canned spinach or beans. Dickson⁸⁰ proved beyond question the ability of *B. botulinus* to form its toxin in canned peaches, apricots, string beans, peas, corn, asparagus, and a medium made of fresh artichokes. Subsequent to this publication much experimental and epidemiologic evidence had accumulated to attest further, if such be necessary, to the correctness of Dickson's conclusions. The known range of substratums which will support the growth and toxin production of *B. botulinus* is constantly being widened by evidence from many sources.

⁹⁰ Hyg. Rundschau, 1904, 14, p. 449.

⁹¹ South. Calif. Pract., 1910, 25, p. 121.

⁹² Arch. Int. Med., 1914, 14, p. 589.

⁹³ Colorado Med., 1917, 14, p. 35.

The growth of *B. botulinus* in various vegetable and fruit mediums in this laboratory has not been uniform. Especially has this been true of spinach, as has been recorded by Schoenholz, Esty and Meyer.⁹⁴

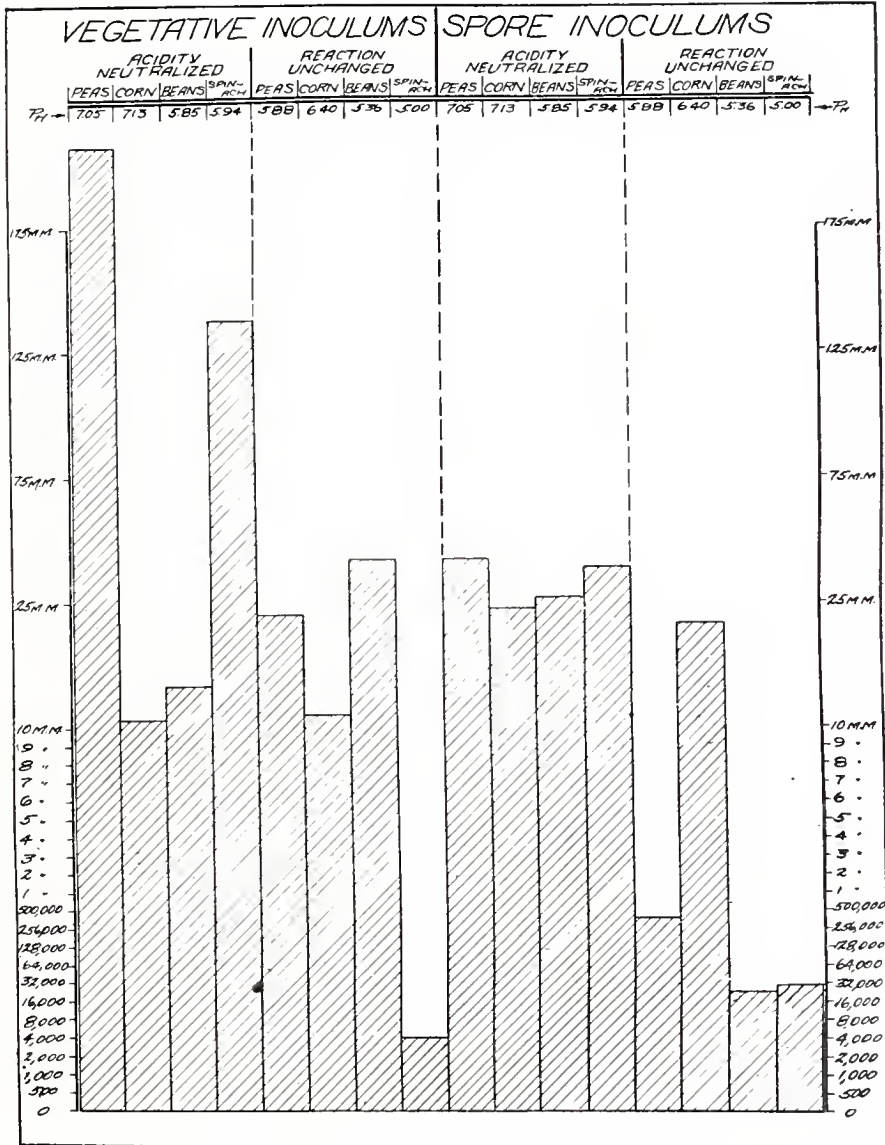


Chart 6.—Growth of strain 97 in vegetable mediums.

It was therefore planned to determine quantitatively the amount of growth supported by peas, corn, string beans and spinach, with reaction unchanged, and in the same mediums with acidity neutralized.

⁹⁴ Jour. Infect. Dis., 1923, 33, p. 289.

Canned vegetables were mashed in their liquor and worked by means of a sterile paddle through two thicknesses of sterile cheesecloth. Every precaution was taken to prevent wide contamination. A mass of the consistency of rather thin cream was secured in each instance, half of which was immediately tubed in 10 c.c. amounts, petrolatum stratified and sterilized by autoclave at 115 C. for 20 min. The P_H of the other half of the 4 liquors was adjusted with normal sodium hydroxide as nearly as possible by the use of indicators to P_H 7, and the same procedure followed as described for the first half. After sterilization the P_H 's were determined in duplicate. The natural reaction of the corn, P_H 6.4, is within the optimum range found for the standard medium. The natural reaction of the peas, P_H 5.88, while not within such optimum zone, is not far removed. The natural reaction of the beans was not far from the limiting acid concentration and that of the spinach, P_H 5, is on or near the threshold. The reactions of corn and pea mediums which had received the neutralizing sodium hydroxide were 7.13 and 7.05, respectively. The beans and spinach, however, were still definitely acid, with P_H 's of 5.85 and 5.94, respectively.

Vegetative Inoculums: One c.c. of an 18-hour culture of strain 97, a type A recently isolated organism, was inoculated into one each of the two sets of mediums, and platings were made after 18 hours of growth at 37 C. As may be noted from chart 6, each of the mediums in which the acidity had been partially neutralized showed growth, ranging from a vigorous proliferation in the peas and spinach to a moderate amount in the corn and beans. In every case except the corn the growth was appreciably less vigorous in the mediums with reactions unchanged, and in the spinach substratum with reaction unchanged no growth was demonstrable. The equality of growth in the two sets of corn medium is not surprising, since the P_H of both was in the optimum zone.

Spore Inoculums: One c.c. of 10-day old strain 97, heated for 1 hour at 80 C., was inoculated into one flask each of the two sets of mediums, and platings were made after 42 hours at 37 C. Macroscopic observation suggested this as the best time, but it is possible that the optimum growth period had been passed. The results, nevertheless, again are evidence of the inhibiting effect of acidity in the spinach with reaction unchanged.

Growth of Strains 38 and 40 in String Bean Medium.—Chart 7 contains data on counts of strains 38 and 40 in 200 c.c. amounts of the natural reaction string bean medium used in the previous experiments. Platings were made immediately following inoculations with 18-hour cultures and after incubation at 37 C. for 18, 24, 36, 48 and 72 hrs. There is revealed a biologic value for this vegetable medium which is in general less than that shown for the veal infusion—2% peptone mediums of the same P_H , but the growth is well maintained during the 3 days of the experimental period.

The results as a whole are evidence of the wide range of acidity tolerated by the organism, and also of the wide range of vegetable substratums which support fair growth of *B. botulinus*. The P_H , 5, of the spinach medium was slightly lower than the lowest recorded by Bigelow and Cathcart,⁹⁵ 5.14, which is undoubtedly due to the acidity developed

⁹⁵ Nat'l Canners Assn. Bull., 17-L, 1921.

during sterilization. They remarked concerning the wide variation in P_H of different samples of spinach, and record figures ranging from 5.14 to 5.74. The acidity of the beans, peas and corn falls within the ranges obtained for commercially canned products by the same workers. The bottles of spinach and beans with spore inoculums and the bottle of spinach with vegetative inoculum which failed to show growth were kept in the incubator for 15 days, and no organoleptic evidence of growth was observed at any time. It is undoubtedly significant that

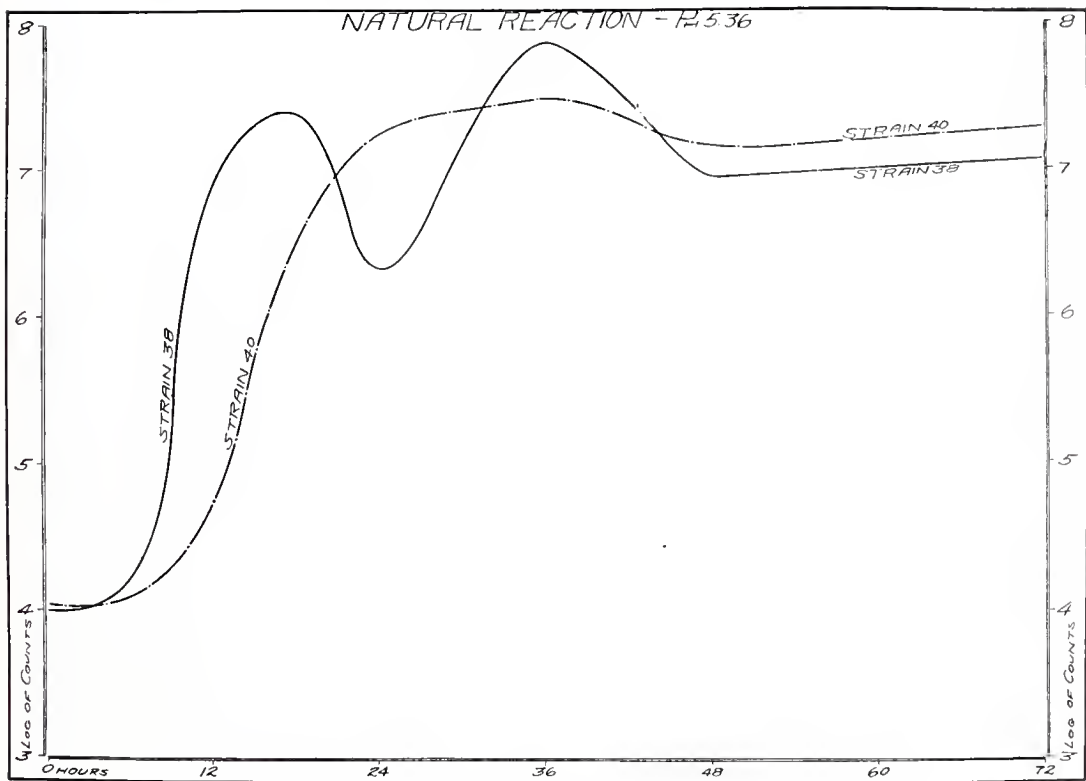


Chart 7.—Growth of strains 38 and 40 in string bean medium.

these 3 bottles were the ones with the greatest acidity. The only bottle of 4 of the bean medium with natural reaction failing to show growth after inoculation was the one planted with strain 97 spores. As has been stated, this is a recently isolated strain, and it seems probable that its range of adaptability to acidity is not as wide as that of older laboratory strains, and therefore more nearly approximates natural conditions. The limiting concentration of hydrogen ions for vegetable mediums is probably nearly, but not quite, as high as for double strength veal

infusion—2% peptone. In fact, it may be stated that *B. botulinus* appears to be a relatively hardy organism, generally growing well on a meager fare if such environmental conditions as acidity, alkalinity, temperature, light and anaerobiosis are favorable. Conversely, the organisms can undoubtedly grow and reproduce with some or perhaps all environmental factors unfavorable if immediately usable food substances are present. In other words, bacteriostasis growth is a reversible equation, and the point of equilibrium is shifted by increasing the sum total of the components of either state.

Compilation of Recent Figures for Aerobic Growth and Comparison with Figures Obtained with B. Botulinus.—As quantitative methods have not been applied before to estimating anaerobic growth, it is of interest to review recent work showing the number, as determined by plating, of viable aerobes which a definite quantity of laboratory mediums can support.

Lane-Clayton⁷⁷ states, "400,000,000 to 800,000,000 per c c. of broth seems to be the maximum for *B. coli* and *B. enteritidis*."

Chesney's work⁹⁶ shows 2,500,000,000 *B. coli* per c c. of bouillon at the height of growth, while pneumococci types I and II numbered 290,000,000, and 180,000,000 per c c., respectively.

Shohl and Janney's⁹⁷ figures for *B. coli* in urine show a maximum of 9,900,000,000 per c c., which is extremely high when compared with other aerobic counts.

Dernby and Avery⁴⁴ report a maximum 18-hour growth of 1,700,000,000 type I pneumococci per c c. of broth, yet the average as judged by the curve presented is between 300,000,000 and 400,000,000.

Cohen and Clark's⁹⁸ curves show 180,000,000 *B. coli* per c c. at the 10th hour of growth; 200,000,000 *B. dysentery* (Flexner); and 230,000,000 *B. aerogenes*, with decidedly lower numbers of *B. proteus*, *B. alkaligenes* and *B. dysentery* (Shiga).

Graham-Smith,⁷⁶ relative to growth of *Staphylococcus aureus*, states the maximum number in meat extract is about 1,000,000 per standard loop, or 1,000,000,000 per c c.

From an average obtained by plating 17 strains of stock cultures of *B. typhosus*, Schoenholz and Meyer¹⁵ have published a curve showing about 1,000,000,000 viable organisms per c c. in a medium identical with my standard.

⁹⁶ Jour. Exper. Med., 1916, 24, p. 387.

⁹⁷ Jour. Urol., 1917, 1, p. 211.

⁹⁸ Jour. Bacteriol., 1919, 4, p. 409.

The numbers of viable streptococci reported by Foster ⁵⁴ (up to 1,040,000,000,000,000 per c.c.) in his standard meat extract medium are extraordinary and undoubtedly contain an error which may well explain his statement, "Attempts to construct growth curves by plating the logarithms of counts against time brought out certain irregularities which made impossible the formulation of smooth curves."

Wilson ⁴⁰ has recently reported growth of *B. suispestifer* in Esmarch tubes up to a maximum of 700,000,000 per c.c.

This cursory survey is reassuring, since it allows the conclusion that the plating method generally recognized as a standard one for measuring aerobic growth is equally applicable as adapted for quantitative estimation of anaerobic growth. Maximum figures reported herein or in preceding publications ³⁴ are in the neighborhood of 300,000,000 to 500,000,000 *botulinus* bacilli per c.c., and the organisms involved are comparatively large. These figures bear very well the criteria recommended by the Progress Report for 1920 of the Committee on Bacteriological Technic.⁹⁹

CONCLUSIONS

The optimum range of hydrogen-ion concentration for *B. botulinus* in phosphate buffered double strength veal infusion-2% Difco peptone is P_H 6.0 to 8.2, inclusive, with a mean near P_H 7.0, when vegetative forms are planted. The limiting range for 3-day growths is from P_H 5 to 9, inclusive. Spore inoculums have an optimum range from P_H 6.0 to 7.2. The indications are that a slight acidity may be a stimulus to spore germination.

Approximately the same optimum zone, P_H 6.0 to 8.9, was demonstrated for *B. botulinus*, *B. sporogenes*, and *B. histolyticus* vegetative inoculums in autolyzed veal infusion.

B. botulinus grows well at 37 C., but the number of viable organisms decreased rapidly at such a temperature. The peak of proliferation is reached somewhat more slowly at 26.5 C., and the decline in numbers is retarded, while at room temperature growth proceeds more slowly still, and the decline in numbers of viable organisms is markedly retarded. The decline in numbers of viable organisms is followed by autolysis, which is probably the mechanism of toxin formation.

Vegetable medium support fair growths of *B. botulinus*. The most active limiting factor in such mediums seems to be the natural acidity.

⁹⁹ Jour. Bacteriol., 1921, 6, p. 135.

INHIBITIVE INFLUENCE OF SUGARS AND SALT ON VIABILITY, GROWTH, AND TOXIN PRO- DUCTION OF *B. BOTULINUS*. XVII

CARRIE CASTLE DOZIER

*From the George Williams Hooper Foundation for Medical Research, University of California
Medical School, San Francisco*

*Aided by grants from the National Canners Association, the Canners League of California
and the California Olive Association*

THE INHIBITIVE INFLUENCE OF SUGARS

Matzuschita¹ reported an optimum of from 2-10% of glucose for the growth of *B. botulinus*, with a maximum of 55%. Dickson, Burke and Ward's² work showed "certain fruits which have been canned in sugar form suitable mediums for the growth of *B. botulinus* and the development of its toxin." They found, "the addition of cane sugar to beef broth in concentrations up to 64% does not prevent the growth of *B. botulinus* or the formation of its toxin." Their criteria of growth after incubation at 30 C. for 3 and 1 months, respectively, were presence of toxin and growth in transplants. Measuring growth by presence of toxin, Thom, Edmondson and Giltner³ report positive findings in cooked meat medium containing 35% of glucose but not in 45%, after an incubation period of 7 days at 37 C. No further evidence was found by me concerning the inhibitive effect of sugars on the growth of *B. botulinus*, although a recent editorial⁴ states: "Growth is hindered by a high concentration of sugar or brine."

EXPERIMENTAL

Weighed amounts of the sugars were introduced into volumetric flasks and made up to volume with standard medium of P_H approximately 7.2. This was then distributed into test tubes in 10 c.c. amounts unless otherwise noted, oil or petrolatum stratified, autoclaved for 20 min. at 115 C., incubated 48 hours for sterility, and immediately before inoculation heated in a boiling water bath for a few minutes. The P_H was always determined from pooled samples before inoculation. Unless otherwise noted, toxin tests were conducted by forced feeding of 2 c.c. amounts to guinea-pigs.

*Growth in Standard Medium Containing 1% to 25% of Glucose or Sucrose (Commercial Cane Sugar).—*Ten c.c. amounts of standard medium containing 1, 5, 10, 20 and 25% of glucose or sucrose, oil stratified, were inoculated with

Received for publication, Jan. 30, 1924.

¹ Arch. f. Hyg., 1902, 43, p. 267.

² Arch. Int. Med., 1919, 24, p. 581.

³ Jour. Am. Med. Assn., 1919, 73, p. 907.

⁴ Ibid., 1922, 79, p. 42.

0.1 c.c. each of an 18-hour culture, or a spore suspension which had just previously been heated 1 hour at 80 C. Spores were used from strains 38 and 42, and vegetative inoculums from 9 strains, a duplicate set of tubes receiving inoculums from strain 38. Macroscopic readings were made daily for 7 days, and in no case did the cultures fail to become distinctly turbid. The higher percentage of glucose seemed to inhibit the growth somewhat, and there was a greater toxin production in the medium containing 25% sucrose than in the 25% glucose-containing tubes. The average time of death of guinea-pigs receiving subcutaneously 1 c.c. of the culture fluids after centrifugation for 1 hour was 45 and 19 hours, respectively.

Before inoculation the glucose-containing mediums showed an increasing acidity paralleling the glucose concentration, which was not found to be the case with the sucrose-containing mediums. This presumptive evidence of the integrity of the sucrose molecules during sterilization is in harmony with the work of Mudge⁵ and of McAlfine,⁶ who found only a slight hydrolysis of sucrose after autoclaving for 1 hour, and none at all after 15 and 30 min. The hydrolysis of glucose during sterilization, resulting in increased acidity, undoubtedly helps to account for the suppression of growth reported by Thom and his co-workers³ in 45% glucose meat infusion, and for the lesser toxin production just reported in 25% glucose standard medium than in that containing 25% of sucrose. Schreiber⁷ could not secure growth of the Milzbrand bacillus with more than 15% glucose, yet Lubbert⁸ succeeded in growing *Staphylococcus pyogenes aureus* in 48% cane sugar. Bitting⁹ was unable to note any inhibitive action of cane sugar on the general aerobic flora of tomato bouillon in less than 25% concentration.

Growth in Standard Medium Containing 25%, 50% and 75% of Sucrose.—
Exper. 1: In this series of tests, petrolatum instead of paraffine oil was used as a more effective seal. Aside from the superior anaerobic conditions maintained by petrolatum seals, their use allows an estimation of the amount of gas produced. The cultures were discarded at the end of 10 days' incubation at 37 C., during which time all of the 25% sucrose cultures had shown definite turbidity and gas formation; 2 of the 50% cultures had been definitely turbid and 2 others showed slight gas production; while the 75% sucrose cultures gave no evidence of growth as measured by turbidity or gas. Toxin tests were positive in 3 cases and negative in 1 for the four 50% cultures which had shown turbidity or gas, or both.

It may here be remarked that with a 75% sugar solution great care must be exercised in securing a good mixture of inoculum and solution. On account of the great viscosity of the latter, unless thorough mixing

⁵ Jour. Bacteriol., 1917, 2, p. 403.

⁶ Abstr. of Bacteriol., 1923, 7, p. 5.

⁷ Centrabl. f. Bakteriolog., 1896, 20, pp. 353 and 429.

⁸ Cited by Gotschlich in Handb. d. path. Mikroorganismen, 1912, 1, p. 105.

⁹ Bull. 119 Bureau of Chem., U. S. Dept. of Agric., 1909.

is secured, the inoculum tends to rise to the top. In fact, the difficulties of manipulation are so great that further work was confined to concentrations of not more than 70%.

Exper. 2: Thirteen 24-hour old cultures were planted in standard medium containing 50% sucrose, and growth as judged by gas production, had occurred in all but culture 80 after 10 days' incubation at 37 C. Culture 53, which had not proved toxic in exper. 1, was not virulently toxic in this series. Cultures 77 and 86 were not toxic in 1 c.c. amounts injected subcutaneously. On the other hand, strain 80 formed no gas and yet proved toxic to the guinea-pig inoculated.

Growth in Standard Medium Containing 40%, 50%, 55%, 60%, 65% and 70% Sucrose.—Thirty-eight strains of *B. botulinus* (with the possible exception of strain 42, which is nontoxic, but resembles *B. botulinus* morphologically and biochemically) and 2 strains of *B. sporogenes* were used in this test. One-sixth c.c. amounts of 18-hour growths were inoculated, and the cultures were incubated for 150 days at 37 C. For the first 2 weeks growth observations were made daily, and then 3 times per week. Growth occurred in the 40% sugar solutions with comparatively little delay, but in 21 of the 50% solutions the appearance of turbidity and gas was delayed up to a maximum of 46 days, and in 17 cases neither turbidity nor gas production was observed during the whole period of incubation.

No effort is made to explain why gas production was more rapid and more general in the preceding experiment. The mediums were as nearly alike as it was possible to make them, but it is probably significant that different lots of double strength veal infusion were used. The peptone was from one bottle. None of the *B. botulinus* cultures grew in the 55% sugars, nor did either of the *B. sporogenes* strains. One of the latter grew in the 50% sugar, but the other did not, which suggests that sucrose is at least as inhibitive for *B. sporogenes* as it is for *B. botulinus*.

Quantitative Results on the Inhibitive Action of Sugar on the Growth of B. Botulinus.—Standard medium containing 0, 10, 25, 40, 60 and 70% of sucrose was prepared and bottled in 200 c.c. amounts, petrolatum stratified, autoclaved 20 min. at 115 C., and incubated 48 hours as a test for sterility. Five c.c. samples removed from like concentrations of sugar in the two sets of bottles just prior to inoculation were pooled and used to determine the hydrogen-ion concentration. There was a gradual decrease in P_H paralleling the concentrations of sucrose, but in no case was the acidity increased beyond the optimum zone. The original standard medium was distinctly alkaline, P_H 7.5, which probably accounts for the breaking down of some of the sugar molecules during sterilization.

One c.c. amounts of 18-hour old strains 38 and 40 were inoculated into sets of these flasks. Samples were removed and plates poured after 18, 48, and 72 hours of incubation at 37 C. Colony growth, from samples taken from the cultures of strain 38, indicates that while 10% of sucrose may slightly retard

growth, it is only temporarily inhibitive; 25% is definitely inhibitive, and figures covering both the 10% and 25% solutions attest to a rapid destruction of the organisms which did grow. Slight proliferation is shown for the 40% solution on the 72nd hour of growth, and figures for the 50% solution do not entirely negative a suggestion of slight proliferation. No growth was demonstrable in the 60% and 70% solutions.

Evidence secured with strain 40 is not essentially different, except that no proliferation is indicated in the 40% and 50% solutions, and counts made on the 120th hour of growth from the 50%, 60% and 70% cultures indicate that most of the organisms inoculated were no longer viable.

Toxin tests were generally corroborative. Two c.c. of strain 38 10-day old centrifuged culture fluids from the flasks containing 0%, 10%, 25% and 40% sucrose given orally to guinea-pigs of approximately equal weights, caused death within 18 hours. Strain 40 cultures tested at the same time were equally toxic for 0%, 10%, 25% and 40% sucrose. The significance of the toxicity of the cultures containing the higher amounts of sugar will be discussed in subsequent paragraphs, but it is here pertinent to note that there is a distinct line of demarcation between the toxin titers of both sets of 40% and 50% cultures. It is not improbable that active proliferation occurred in the 40% strain 40 tubes after the third day of incubation, although at no time up to 2 weeks was a definite turbidity observed. The integrity of the petrolatum seal had, of course, been violated during sampling, and its use as an indicator of gas production thereby destroyed.

RELATIONSHIP OF GROWTH AND TOXIC PRODUCTION

Further investigation of the relationship between growth and toxin production was desirable. A repetition of the immediately preceding experiment was undertaken, with the exception of the plate counts. The results are strikingly similar to those obtained before. No evidence of gas formation or turbidity was secured in the 60% and 70% sucrose cultures, but after an incubation period of 14 days 2 c.c. of the supernatant fluid from strain 38 cultures were lethal for guinea-pigs in 72 and 48 hours, respectively, while the 50% cultures, in which there had been definite turbidity and gas production, were perhaps slightly less toxic. With strain 40, 2 c.c. of the 50%, 60% and 70% solutions were lethal in 13, 13, and 16 days, respectively.

A third test was made on a set of the same mediums, using as inoculums a 16-hour culture of strain 38. Immediately after planting a sample was removed from each bottle, centrifuged, and 2 c.c. fed to guinea-pigs. The pigs which received the feedings from the 0%, 10%, 25% and 40% solutions were alive and well when discarded 30 days later. The pig which received the 50% sample died on the 15th day after feeding, but necropsy findings were suggestive only for botulism. It cannot be denied, however, that this may have been the cause of death. The pig which received the 60% feeding died on the 7th day from a frank paratyphoid infection, while the one which received the 70% feeding was alive and well on the 30th day after the feeding. The cultures were incubated 19 days at 37 C. and the toxicity again tested by feeding. Two c.c. from the cultures in which there had been definite evidence of proliferation proved lethal in 12 hours or less; while the 50% and 60% cultures, in which there may have been slight proliferation (no gas, but turbidity questionable), were lethal in 24 hours or less; and, finally, the 70% culture in which no evidence of proliferation could be secured, was lethal in 33 hours.

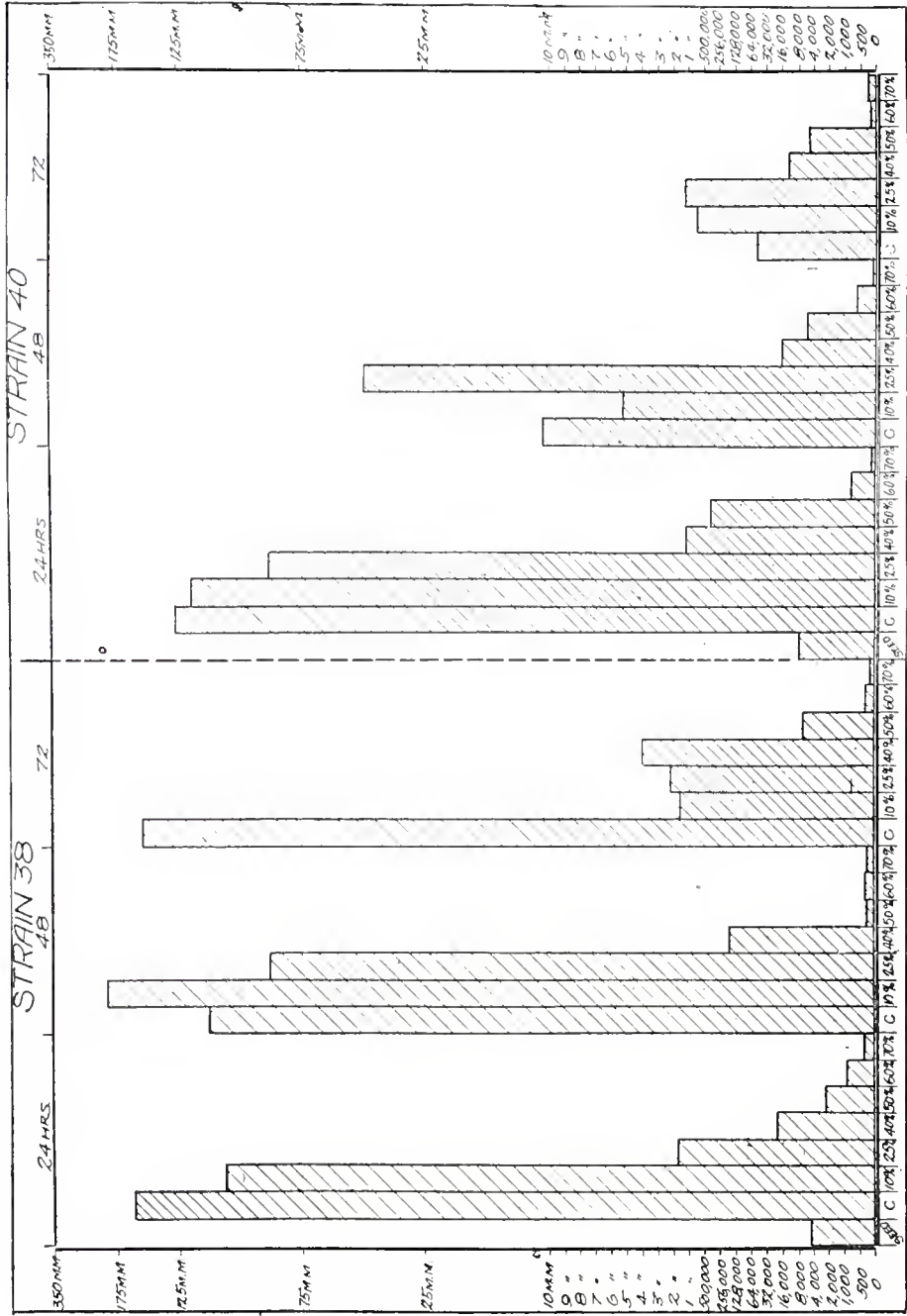


Chart 1.—Growth in standard media with from 10 to 70% of sucrose.

Spore Inoculums.—The results reported above were next verified with detoxified spore inoculums. Spore suspensions of strains 38 and 62 were heated for 1 hour at 80 C. and inoculated into sets of sugar-containing mediums identical with those used for vegetative inoculations. Again the results were similar to those reported above. In no case was there death of animals fed the centrifuged supernatant fluids immediately after inoculation; and in no case was there survival of the test animal after taking 2 c.c. of the supernatant fluid from the cultures incubated for 19 and 14 days, respectively, and again there was a clearcut line of cleavage between the toxin titers of the 40% and 50% cultures. Two c.c. of strain 38 centrifuged fluid from the spore suspension fed to a pig proved lethal in 12 hours, but after heating for 1 hour at 80 C. prior to inoculation, its toxicity could no longer be demonstrated by feeding. The first test after heating was made with undiluted culture fluid and with a 1:200 dilution in physiologic saline, the latter making a strength comparable to that of the culture mediums after inoculation. Unfortunately, the pig which received the 2 c.c. of undiluted fluid met with a death which from circumstantial evidence was pronounced accidental. Since some of the original spore suspension was still available, a portion was heated for 1 hour at 80 C., centrifuged, and the feeding tests repeated. Neither the undiluted fluid, nor that given in a 1:200 dilution proved toxic.

Unheated, the supernatant liquor of strain 62 spore suspension proved toxic when given orally and when injected subcutaneously. Heating for 1 hour at 80 C. may have left undestroyed a little of the toxin, since the pig which received 2 c.c. orally succumbed in 9 days, and necropsy findings were inconclusive. Physiologic saline dilutions of 1:10 and 1:100 were nontoxic.

INHIBITIVE INFLUENCE OF NaCl

Karaffa-Korbutt¹⁰ published a concise review of the literature and reported experimental work on the effect of salt on bacterial viability and proliferation. He found concentrated salt solutions killed spore-free bacteria in 2 to 3 months, but spores were more resistant. Stadler¹¹ reviewed Forster and de Freytag's results which definitely differentiated between the resistance of spores and vegetative forms of *B. anthrax*. The former they found viable after 6 months' suspension in concentrated salt solutions, while the latter were not viable after 2 hrs.

The earliest record of the effect of salt on *B. botulinus* is that by van Ermengem,¹² who found no growth of the Ellezelles strain in glucose bouillon or gelatin with 2% salt, although it should be noted that the medium he recommended contained 1% salt. He found growth greatly inhibited in pork infusion when the salt reached a concentration of 5%, and in 6% concentration he was not able to detect any growth. He therefore considered meat pickled in 10% brine safe. This conclusion also appears warranted from the results of Matzuschita,¹ who

¹⁰ Ztschr. f. Hyg. u. Infektiöskr., 1912, 71, p. 161.

¹¹ Arch. f. Hyg., 1899, 35, p. 40.

¹² Handb. f. path. Mikroorganismen, 1913, 4, p. 909.

found 7% the maximum concentration for growth. Tanner¹³ quotes Stadler¹¹ as having found an inhibition of *B. botulinus* in 7-10% of salt, but this could not be confirmed. Armstrong, Story and Scott¹⁴ report growth of *B. botulinus* in meat infusion-dextrose medium containing 6% NaCl, while Thom, Edmondson and Giltner,³ using the Nevin strain, found growth and toxin production in cooked meat medium containing 5% of NaCl, but not in 8%. Wyant and Normington's¹⁵ conclusion is that growth often occurs in bouillon mediums which contain up to a maximum of 10% salt. The value of this work is much weakened because of lack of evidence as to purity of cultures used.

EXPERIMENTAL

The salt used was from the same box of a chemically pure product, and the manner of preparation of the mediums was in all respects similar to that described for sugar-containing mediums. The addition of various amounts of salt exerted little effect on the reaction of the standard medium. Oil stratification was practiced, at first, but later petrolatum seals were used. Unless otherwise noted, transplants were made into 10 c.c. amounts of van Ermengem medium, prepared according to the modifications described by Dubovsky and Meyer.¹⁶

Growth in Standard Medium Containing 2% of Glucose and from 1% to 8%, Inclusive, of NaCl.—Exper. 1: Inoculations of 0.1 c.c. of 24-hour cultures of 14 strains of *B. botulinus*, and 1 strain each of *B. tetani*, *B. histolyticus* and *B. sporogenes* were made into mediums with 0, 1, 2, 3, 4, 5, 6, 7, and 8% NaCl. Observations were made daily for 7 days, at the end of which time *B. botulinus* strains 4 and 21 had not shown growth in the 4% solutions; but the latter had grown in the 5% tube. Five of the 14 strains had not grown in the 5% solution, while none had shown any growth in the 6% and 7% tubes. Atypical strains 42 and 45 showed growth in the 8% solution. The strain of *B. tetani* behaved very much like the average for the *B. botulinus* cultures, while *B. histolyticus* grew vigorously in all concentrations of NaCl, and *B. sporogenes* failed to grow in the 6% and 8% solutions, but did grow in the 7%.

Microscopic Pictures of Growth.—Gram stains of the *B. botulinus* organisms from tubes in which growth had occurred were made on the days when subcultures were made from tubes not showing growth. The most outstanding feature of the series is the lack of uniformity of the microscopic pictures presented by the various strains. Only two strains, 21 and 56, type A organisms, showed, when examined, consistent sporulation in all the various salt concentrations in which growth

¹³ Bacteriology and Mycology of Foods, 1919, p. 500.

¹⁴ Pub. Health Rept., 1919, 34, p. 2877.

¹⁵ Jour. Bacteriol., 1920, 5, p. 553.

¹⁶ Jour. Infect. Dis., 1922, 31, p. 501.

was evident. No spores appeared in the stronger salt solutions in which visible growth had not occurred, but wholly satisfactory stains were not secured. It is evident that under the conditions of the experiment, increasing salt concentration neither favors nor completely inhibits sporulation of *B. botulinus*. It is equally evident that *B. botulinus* may form spores without the continuous alkaline reaction, which von Hibler¹⁷ found a most desirable condition for the sporulation of anaerobes, including *B. botulinus*. The salt seemed to exert a specific effect on the bacilli, which consistently decreased in size with the increasing salt content of the mediums.

Strain 37 was planted in duplicate and one set examined on the 7th day of incubation and the other on the 30th day. It is of interest to note that the microscopic pictures of like concentrations were almost similar on the two days, suggesting a permanence of the spores and not a temporary sporulation such as von Hibler found for several anaerobes in weakly alkaline and not completely carbohydrate-free mediums.

With strain 9, microscopic findings corresponded with cultural results in that this strain was the first one to show beginning loss of viability in the higher salt concentrations on the 30th day of incubation, and the only one to show definitely disintegrated forms and a completely gram-negative picture in tubes showing growth, indicating a protoplasmic deterioration. The distinctly acid reaction of these tubes undoubtedly is related to the disintegration of the bacilli, but the cause of the acidity is not evident. That it is a direct result of the salt content is indicated by the consistent increase of acidity with increase of salt concentration, and one may assume that it is not a direct function of the amount of growth, which could hardly be more profuse in the 5% salt concentrations than in the 2%. On the other hand, the acidity of the 7% tube, in which viable organisms could not be demonstrated, was approximately the same as that of the 6% and 8% tubes, from which transplants grew vigorously. Strains 42 and 45, which have been described as "atypical," proliferated in the 8% salt solution, but showed a different microscopic picture. Strain 42 organisms were distinctly smaller than ordinary, but no involution forms were found, while strain 45 had responded to the 8% salt content by the production of aberrant atypical rods. Subcultures from 6% and 7% salt cultures of this strain failed to show any viable organisms.

¹⁷ Untersuchungen über pathogene Anaeroben, 1908, p. 185.

Exper. 2: The essentials of the first part of exper. 1 were repeated, using, however, only 4 of the strains and making the tests in triplicate. Better anaerobiosis was insured by the use of petrolatum seals. The lower salt concentrations which had shown vigorous growth were omitted and concentrations up to and including 12% were used.

Strain 45 grew in the three 6% tubes, and in one 7% tube. Strain 6 grew vigorously in the three 5% tubes, and not at all in the higher concentrations; while strains 38 and 40 showed considerable variability. Two of the former grew vigorously in the 4% tubes, and one of these sparsely in a 5% tube, while the third 4% and the remaining 5% tubes planted with strain 38 gave no evidence of growth. Approximately the same was true with strain 40, only one of the three 5% tubes showing any evidence of proliferation. Subcultures were made on the 10th, 20th, 30th, and 60th days of incubation from a set of tubes planted with each strain in which no growth had been demonstrable. With strain 6 this began with the 6% tubes; with strains 38 and 40, all the tubes, including the 4% were subcultivated; while the strain 45 subcultivation began with the 7% tube. Strain 45 differentiated itself again from the other strains in showing active growth in the highest salt concentration and in not remaining viable in the salt solutions in which proliferation had not taken place; that is to say, not one of the 10-day subcultures from strain 45 tubes grew. Subcultures from the other strains on the 10th, 20th, and 30th days of incubation all grew, but on the 60th day strain 6 organisms in the 7% to 12% tubes were no longer viable; the 6% tube of strain 38 was the only one which failed to show viable organisms, while no growth was secured from any of strain 40 tubes.

Growth in Standard Medium Plus 2% Glucose and from 4% to 9% NaCl., Petrolatum Stratified.—One-tenth c.c. amounts of 24-hour cultures of 7 strains of *B. botulinus* and 2 strains of *B. sporogenes* were introduced into petrolatum stratified standard medium containing 2% of glucose and from 4% to 8%, inclusive, of NaCl. In every case vigorous growth was observed in the 4% and 5% solutions, but the 6% solutions were partially inhibitive, except in the case of one of the strains of *B. sporogenes*, and in the case of *B. botulinus* strain 63 proliferation was questionable. In the 7% solution, *B. botulinus* strains 79 and 45 showed proliferation by turbidity and gas, while with strains 77 and 86 no turbidity was observed, but there was a definite but small amount of gas formed. The *B. sporogenes* strains both grew actively in the 7% and 8% solutions, but not in the 9%. No *B. botulinus* strain showed any evidence of growth in the 8% or 9% tubes. The results are indicative of the better conditions for growth afforded by the petrolatum seals than the layers of paraffin oil.

Viability After 10 to 60 Days in Standard Medium Containing 10, 15, 20 and 25% NaCl.—Mediums were prepared with 10, 15, 20 and 25% of NaCl and oil stratified. Ten c.c. amounts of these were inoculated with 0.1 c.c. of 24-hour cultures of 11 strains and spore suspensions of 2 strains. No visible growth appeared in any of the tubes. Transplants into van Ermengem medium made on the 10th day of incubation showed viable organisms present in every case. The same was true of 20 and 30-day transplants. There is not enough evidence as to variations in inhibition of the different concentrations to warrant any conclusions as to relative toxicity of the different concentrations of salt, but the fact that in 3 out of the 5 cases in which 1 c.c. subcultures failed to grow after 60 days' incubation, the 10% solutions were concerned, and in no case did the 25% solutions prove lethal, suggests that osmotic changes alone are not responsible for the injuries sustained. The relation of salt content of mediums to spore formation will be discussed in subsequent pages, but these results do not sug-

gest any stimulation of true sporulation. Even assuming the presence of a few spores in the original inoculums, these results are not comparable with the results of Esty and Meyer,¹⁸ who found the resistance increased by incubation for 60 days in 20% salt solutions, since they used the more sensitive peptic digest-beef heart mince for subcultures, and their spores were incubated in sealed tubes. The inadequate protection provided by paraffin oil stratification was not at the time fully appreciated, and it undoubtedly accelerated the destructive effect of the salt solutions. The results clearly indicate, however, that the higher concentration of salt were no more toxic than were concentrations quite near the borderline of inhibition.

Cultures from the transplants from higher salt solutions were made in liver-glucose agar and colony form noted. In no case could it be determined that the organisms had suffered any hereditary degenerative changes, as evidenced by atypical colony growth, from the long sojourn in the concentrated salt solutions. The appearance of the colonies was noted as "questionable" and "not quite typical" in two cases, but the variations were within the limits found for different cultures of *B. botulinus*. The toxicity of the supernatant fluid from transplants proved the presence of *B. botulinus* in every case except with 3 type B. strains, which are not generally as toxic as type A, and these were tested in 0.01 c.c. amounts only. Strain 42 was not toxic, but it has already been described as "atypical" in that respect.

Growth and Viability of Spores in Standard Medium Containing 2% of Glucose and 4% to 12% Inclusive of NaCl Petrolatum Stratified.—One tenth c.c. amounts of spore suspensions just previously heated for 1 hour at 80 C. were introduced into 10 c.c. amounts of standard medium containing 2% of glucose and from 4% to 12%, inclusive, of NaCl. Strain 6 grew vigorously in the 4% solutions, but no growth was observed in the higher concentrations. Strain 38 grew in 2 of the 6% tubes, and a 3rd in the 5% only, as did strain 40. Strain 42 showed growth in the 6% tube, as did strain 46, but the latter failed to develop in the 5% tube.

Although spores were not numerous in the strain 45 inoculum, their presence was demonstrated microscopically, yet no growth occurred in any of the salt mediums. No viable transplants were secured after 10 days in the various salt mediums, and the presumption is that the heating at 80 C. for 1 hour had destroyed the organisms. This supposition is considerably strengthened by the fact that salt has never been as inhibitive to the vegetative forms of this strain as it has been to most of the other strains, and therefore growth would have been expected in 4%, 5%, 6%, and possibly in the 7% salt mediums. It will be remembered that this strain has never retained viability after 10 days' incubation in comparatively low concentrations of salt. One is reminded of the inability of van Ermengem's Ellezelle strain¹² to withstand temperatures of this degree. The lack of heat resistance of *B. botulinus* cultures reported by Hall¹³ and by Esty and Meyer¹⁸ may be of a different category in that no spores were demonstrable in their cultures.

Transplants made from the salt mediums in which no visible growth had occurred on the 30th day of incubation showed beginning deterioration with strain 6, which had not been demonstrable on the 10th and 20th days of incubation. On the 60th day, strain 6 organisms were no longer viable, and strains 38 and 40 began to show the effect of the salt, while the viability of strain 46, under the conditions of the experiment, had been destroyed completely sometime during

¹⁸ Jour. Infect. Dis., 1922, 31, p. 650.

¹⁹ Ibid., 30, p. 445.

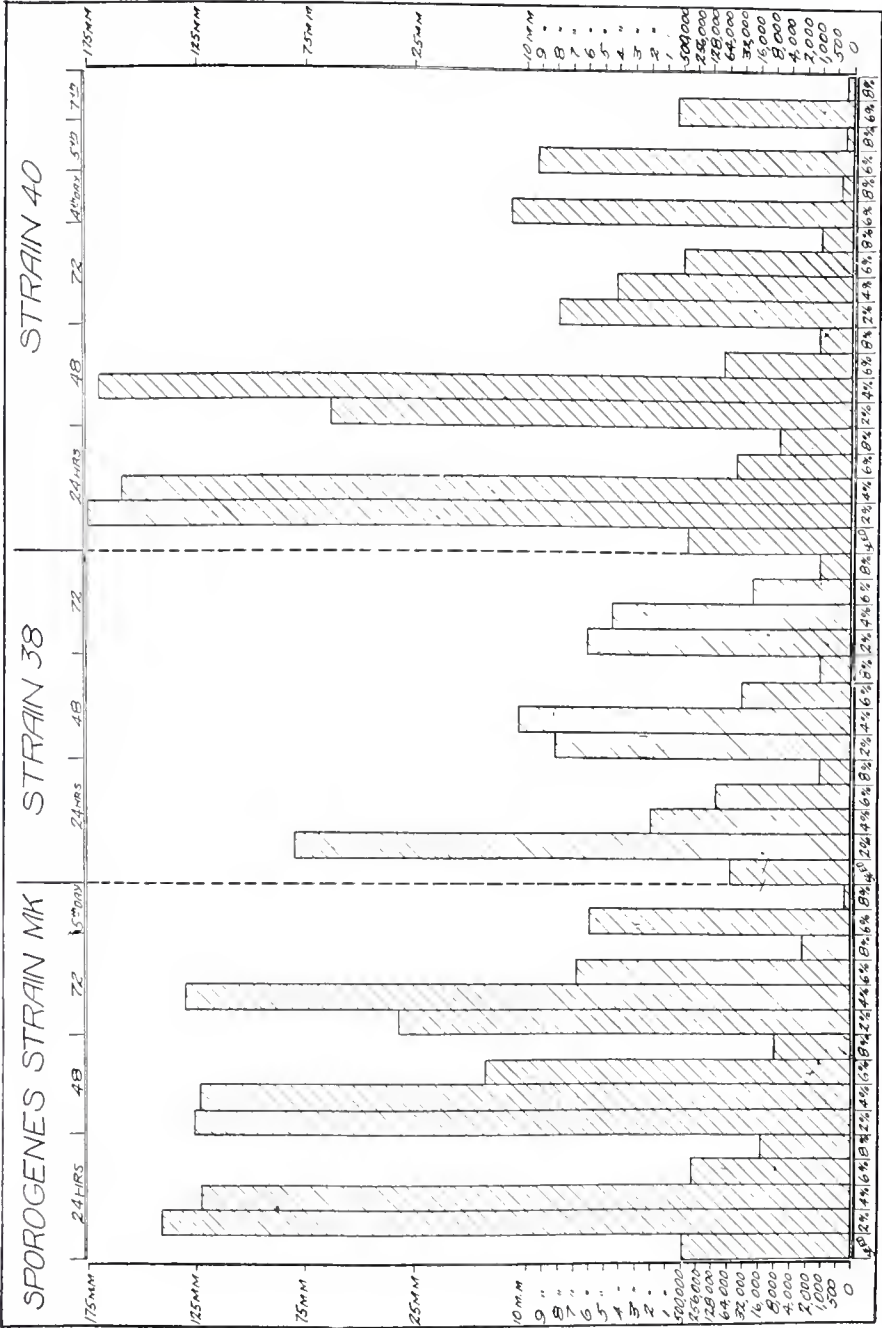


Chart 2.—Growth in standard mediums with from 2 to 8% of NaCl.

the 30th and 60th days of incubation. Strain 6 transplants carried comparatively few spores, but the transplants from strain 46 tubes contained in the neighborhood of 2,000,000,000 spores. The evidence is suggestive of the comparatively equal sensitiveness of heated spores and vegetative forms to medium concentrations of NaCl under the conditions of the experiment. This is very different from the effect of heat alone, to which the spore is many-fold more resistant.^{18, 20}

Quantitative Results on the Inhibitive Action of NaCl on the Growth of B. Botulinus.—Standard medium containing 2, 4, 6 and 8% of NaCl was prepared and bottled in 200 c.c. amounts, petrolatum stratified, autoclaved 20 min. at 115 C. and incubated for 48 hours as a test for sterility. Five c.c. samples removed from two flasks with like concentrations of salt were pooled and used to determine the hydrogen-ion concentration in the hydrogen electrode. In no case did the addition of salt markedly change the reaction of the medium. One c.c. of 18-hour strains of *B. botulinus* 38 (duplicate sets, but inoculated on different days) and 40, and *B. sporogenes* strain MK were introduced into the bottles from which samples were removed and plates poured after 24, 48 and 72 hours of growth. Counts were also secured on the 5th day of incubation of the *B. sporogenes* cultures, and on the 4th and 7th days of *B. botulinus* strain 40 cultures.

Results with *B. botulinus* show that 2% of salt exerts little or no measurable effect on proliferation; a definite and measurable inhibition is shown by plate counts from the 4% solutions, while 6% apparently allowed no growth of strain 38 in 3 days. The 4th day's count of strain 40 shows a delayed slight proliferation, which was no longer demonstrable on the 7th day. The *B. sporogenes* 6% culture showed an increase in viable organisms between the 24th and 48th hours of growth from 323,500 to 18,725,000. No proliferation was demonstrable in the 8% solution in 72 hours. These counts are quantitative confirmation of results with less precise methods. These data are represented graphically in fig. 2.

Relationship Between Growth in Standard Medium Containing 4% to 20% NaCl and Toxin Production.—Toxin production had not paralleled demonstrable growth in sugar-rich mediums, and like tests were undertaken with substratums containing various percentages of NaCl. Standard medium containing 4, 5, 6, 7, 8, 10 or 20% NaCl was bottled in 30 c.c. amounts, petrolatum stratified, sterilized, incubated for 48 hours, and planted with 1 c.c. of spore suspension which had been heated for 1 hour at 80 C. The toxin tests were conducted by forced feeding. An attempt was made to feed 2 c.c. of the supernatant fluid after 1 hour's centrifugation at high speed, but the pigs objected strenuously to taking the salty fluid, and it is probable that at least one-third of the material was lost in each case. At any rate, never more than 2 c.c. were ingested. The experimental animals were kept under observation for 30 days. Results will be presented as expers. 1, 2 and 3.

Exper. 1: Samples were removed immediately after inoculation with a spore suspension of strain 38, centrifuged and fed. No toxic effect was demonstrable except in the case of the 8% solution. The pig which received this died on the 16th day following ingestion, and the necropsy findings were not entirely negative for botulism, although the preliminary symptoms which are usually present in such delayed onset were not apparent. There was visible growth in the 4, 5 and 6% solutions before the 15th day of incubation. The 4% and 5% tubes were discarded; the 6% tube furnished a fluid which killed the pig in less

²⁰ Burke: Jour. Am. Med. Assn., 1919, 72, p. 88. Orr: Jour. Med. Res., 1920-21, 42, p. 127.

than 18 hours, but the other tubes in which no growth had been observed were nontoxic.

Exper. 2: The test was repeated with strain 97 spore suspension. All the pigs which received the cultures 1 hour after inoculation remained well. After 15 days' incubation, visible growth had occurred in the 4, 5 and 6% tubes, and the first 2 were again discarded. The 6% tube held toxic liquid, as did the 7% tube. The 8 and 20% tubes were nontoxic, but the 10% culture caused the death of a 775 gm. guinea-pig in 5 days. Because of the inconstancy of the results and because of their variation from those secured in the preceding experiment, the feeding was repeated 9 days later, during which time the cultures had stood at room temperature. The toxin titers seemed to have increased somewhat during this period, although the use of much smaller animals makes impossible any direct comparison. The 6 and 7% cultures again killed the pigs within 18 hours; the 8% tube was nontoxic; and the 10% tube was lethal in 48 hours, as was the 20% solution, which had been nontoxic before.

Exper. 3: A part of the spore suspension used for the preceding experiment was centrifuged, the sediment mixed with sterile washed sand and quickly dried in vacuo over concentrated H_2SO_4 in a slightly warmed environment. Just before inoculation an amount of physiological salt solution equal to the original culture liquid was added. This suspension was heated 1 hour at 80 C. just prior to inoculation. Guinea-pigs fed approximately 1 hour after inoculation remained well, with the exception of the one receiving the supernatant liquid from the 8% culture, which died in 24 hours, but necropsy findings were entirely negative for botulism. After 15 days' incubation at 37 C., supernatant fluid from the cultures was again fed and proved decidedly toxic in every case. After standing 9 days at room temperature, another feeding test was undertaken, and the results entirely confirmed the previous findings.

Toxin Production by Vegetative Forms of B. Botulinus in Nonnutritive Substratum.—In order to throw some light, if possible, on the question of toxin production in standard mediums containing high percentages of sucrose and NaCl, an investigation of the possibility of toxin production in nonnutritive substrata was undertaken. Preliminary experiments, as had been anticipated, failed to reveal any visible proliferation of *B. botulinus* in nitrogen-free substrata (distilled water sugar solutions), but no toxin tests were made. Vegetative forms grown in standard medium were centrifuged and twice washed with comparatively large quantities of physiologic salt solution of P_H 7.2, and resuspended in equal amounts of distilled water, distilled water containing 50% of sucrose, and distilled water containing 20% of NaCl.

Exper. 1: A 24-hour culture of strain 38 was used. Immediately after resuspension, samples were removed, centrifuged, and 2 c.c. amounts of the supernatant fluid fed to guinea-pigs of approximately equal weight. The third wash water was nontoxic, the distilled water was lethal in 9 days, and the 60% sucrose and 20% NaCl solutions within 42 hours. After 7 days' incubation at 37 C., the feeding was repeated using pigs of slightly larger size. The intervals before death supervened were, respectively, 5 days, 20 and 20 hours, indicating a slight increase in toxin titers.

Exper. 2: The tests were repeated, using, however, a 21-hour culture of strain 38. This time the second wash water was fed and proved lethal in 39 hours, while the distilled water, the sucrose and NaCl solutions 1 hour after inoculation were lethal in 37, 16 and 15 hours, respectively. After a week's incubation at 37 C., the toxin had deteriorated somewhat, the experimental animals dying after 8 days, 18 hours, and 4 days, respectively.

Exper. 3: A 16-hour culture of strain 97 was used for this experiment. The third wash water was decidedly toxic, causing the death of a 400-500 gm. guinea-pig in 24 hours. Samples from the distilled water, sucrose and salt solutions were lethal in 6 days, 30 and 36 hours, respectively, while after a week's incubation at 37 C., no toxicity was demonstrable.

DISCUSSION

The Inhibitive Influence of Sucrose and NaCl on the Growth and Viability of B. botulinus.—The inhibitive influence of sugars and sodium chloride on the growth of *B. botulinus* and other anaerobes has been measured macroscopically by turbidity; gas formation has been determined by means of petrolatum seals, and finally a quantitative approach to the question has been made by the use of sheep's blood-peptic digest-beef heart infusion-agar plates incubated in safe and efficient anaerobe jars.²¹ Toxin tests have been made, and the results compared with these broadly applicable means of detecting bacterial reproduction.

In no case did 40% of sucrose inhibit proliferation in double strength veal infusion-1% peptone mediums, nor did it greatly retard the appearance of turbidity and gas; but 50% of sucrose inhibited the appearance of turbidity and gas for 150 days in 45% of 38 trials, and retarded their appearance up to a maximum of 45 days in the tubes in which growth occurred. In no case did distinct evidences of growth appear in standard medium containing 55% or more of sucrose. Plate counts have confirmed these qualitative indications that the limiting concentration of sucrose is between 50 and 55%.

The borderline of salt concentration was found by turbidity and gas tests to lie around 6%. This was confirmed by plate counts. With atypical strains 42 and 45, growth occurred in 7 and 8% salt mediums. Strain 42 is of questionable repute, but strain 45, a type A organism isolated from pickled olives, has always been definitely toxigenic. Growth never occurred in concentrations greater than 8%, and in this concentration the toxic effect of the salt was evidenced by the production of pleomorphic vegetative forms. In a series of triplicate cultures with vegetative inoculums, only one showed any turbidity or gas production in the 7% concentrations. With 2% glucose present, 3 out of 7 cultures, including strain 45, showed either turbidity or gas in 7% solutions. No difference in results could be observed when spores were planted, except that growth was never observed in 7% solutions. This lack of suppression of growth by concentrations of NaCl up to and including 6%

²¹ Jour. Infect. Dis., 1922, 31, p. 617.

may well be correlated with the conclusion of Esty and Meyer¹⁸ that no reduction in heat resistance of spores suspended in double strength veal infusion is exerted by salt until the concentration exceeds 6%.

The effects of salt on viability are extremely interesting. No evidence of greater destructive power was secured for the more concentrated salt solutions for either vegetative or moist spore forms over those bordering on concentrations permitting growth. The comparatively great resistance shown by vegetative forms to the anticipated toxic effects of strong salt solutions recalls the relationship which has been observed by various workers between unfavorable mediums and sporulation. Buchner²² found 2% salt distinctly favored sporulation of the Milzbrand bacilli; Matzschita²³ tested many species of bacteria, and concluded that there is a species specificity in the reactions of organisms to mediums containing various amounts of NaCl. Some did not produce involution forms with an NaCl concentration of 10%, while others responded by the production of such forms in the presence of a much lower concentration. In a subsequent publication, he¹ reports the growth of *B. botulinus* in 7% NaCl, but no sporulation in a concentration greater than 5%. Schreiber⁷ found spore formation accelerated when vigorously growing vegetative forms are removed from a medium of good biologic quality and plunged into water or salt solutions. Buchner²² also found that distilled water hastened sporulation.

Microscopic examinations of the organisms planted in the more concentrated salt solutions were frequently made, but the salt content interfered with staining processes, and the presence of other work made an extended examination impossible. However, supplementary tests in which 18-hour standard medium cultures were centrifuged and the organisms introduced into concentrated salt solutions and after 10, 20, 30 and 60 days Gram stained, gave fragmentary evidence of the presence of granular forms, but not of spores. It seems possible that the organisms plunged into a medium with a salt content concentrated enough to prevent active proliferation respond to the osmotic effects by a concentration of the protoplasm which endows them with increased resistance. This supposition is further suggested by the thermal death point studies of such suspensions of Esty and Meyer, who will show the retention of a resistance of more than 5 min. to 100 C. after incubation periods in concentrated salt solutions of 340 days. Their suspensions were preserved in sealed tubes, which undoubtedly accounts

²² Centralbl. f. Bakteriöl. u. Parasitk., 1890, 8, p. 1.

²³ Ztschr. f. Hyg. u. Infektionskr., 1900, 35, p. 495.

for the slower destructive action of the salt than is shown to be the case in the experimental work herein reported. As has been noted, my cultures were incubated under paraffin oil, and a loss of viability began to be manifest by the 60th day of incubation in numerous instances. The lower biologic value of van Ermengem medium used for subcultivating also helps to explain the quicker loss in viability.

Of especial interest is the beginning loss of viability between the 30th and 60th days of incubation of spore suspensions. The results again differ fundamentally from the spore stability, and indeed, the increased resistance to heat which Esty and Meyer¹⁸ found in spores suspended in 20% sodium and potassium chloride solutions after a 37 C. incubation period of 60 days, and the remarkable finding of considerable heat resistance after 340 days in the same salt solutions at the same temperature. Several contributing factors in addition to the two already mentioned in connection with the suspensions of vegetative forms appear to be concerned. The preliminary treatment of the spores has had most influence perhaps. The spores which showed considerable loss of viability on the 60th day of incubation were heated for 1 hr. at 80 C. just prior to inoculation. It seems quite likely that the heat may have altered the permeability of the spore membrane sufficiently to allow the deleterious salt particles freer access to the bacterial protoplasm. This suggested lability of the spores is in all probability not entirely unrelated to the fact disclosed by Fiscoeder's²⁴ experiments. He found that germination of spores sets in much more quickly than formerly was thought. Milzbrand spores transplanted into culture mediums undergo changes within a few minutes which rob them of their heat resistance.

It should also be mentioned that when the experiments here outlined were undertaken, the extent to which *B. botulinus* spores suspended in the original culture fluids deteriorate with increasing age at incubator or room temperature was not fully appreciated, nor had a medium been evolved which consistently produced vigorous spores.¹⁸

Mention has been made of the striking loss of viability of strain 45 in comparatively dilute and in high concentrations of sodium chloride, and the probable sensitiveness to heating at 80 C., in which characteristic it closely resembles van Ermengem's²⁵ Ellezelle strain. It should probably be emphasized that all the purity tests employed in this laboratory¹⁶ have not suggested a contaminating organism.

²⁴ Centralbl. f. Bakteriöl., I, O., 1909, 51, p. 320.

²⁵ Ztschr. f. Hyg. u. Infektionskr., 1897, 25, p. 1.

Slight evidence of an adaptive power on the part of 8 strains of *B. botulinus* was secured by growing them in petrolatum stratified standard medium with an increasing salt content. Beginning with a 3% NaCl concentration, each strain was cultivated for at least 20 transfers in mediums whose salt content was increased by 1% over the previous set. After the 6% concentration had been reached, however, the time necessary for fair growth, as evidenced by gas and turbidity, was so prolonged that it was difficult to keep the experiment moving. Growth in several of the 7% tubes, however, affords evidence of a slight adaptive power of the organisms to increasing salt content.

To summarize briefly: No strain of *B. botulinus* proliferated actively in standard medium containing more than 8% of NaCl or 50% sucrose. With most of the strains, no growth occurred in NaCl concentrations greater than 6%. Heated spores and vegetative forms, under the conditions of the experiments, began to lose their viability in salt solutions in which growth had not occurred after an incubation period of 30 to 60 days. One strain of *B. botulinus* was encountered whose spores seemed to have no resistance to heat for 1 hr. at 80 C., nor to salt solutions which inhibited growth.

The Relationship of Growth to Toxin Production.—Little difficulty was experienced in securing evidence of toxin production whenever visible growth had occurred. No attempt was made to determine the toxin titers of cultures in mediums with the different salt or sugar contents, but death of the experimental animals receiving by pipet 2 c.c. of the supernatant fluid from cultures with 0%, 10%, 25%, and 40% of sugar occurred in general within 18 hrs.; 50% sugar content caused a distinct inhibiting influence on the appearance of visible evidences of growth, and the various strains of *B. botulinus* showed considerable variation in ability to proliferate in that concentration; furthermore, toxin tests were corroborative in that they reveal considerable variation in potency in 50% culture fluids, suggesting various amounts of growth. No growth was observed in standard medium containing 55% or more of sucrose. In a 60% concentration only once was there any indication of proliferation, and in this case the evidence for growth was only a questionable faint turbidity; in the light of further experimental work little weight can be given this isolated bit of personal opinion. On the contrary, toxin was present uniformly in mediums containing up to and including 70% of sucrose when inoculated with *B. botulinus* and incubated 10 to 30 days at 37 C. and at room temperature.

This was true whether vegetative or spore suspensions were introduced. In fact, the results are even more uniform when the latter were planted.

Young vegetative forms, washed two and three times and resuspended in distilled water, distilled water containing 60% of sucrose and distilled water containing 20% NaCl also showed toxin increase, and while the details of the experiments are not altogether uniform, the apparent discrepancies are in general subject to reasonable explanation. A demonstrable amount of toxin has never been found in cultures incubated at 37 C. in less than 14 hrs., and ordinarily even with the very best mediums the toxin titer of a culture begins to be demonstrable only after about the 18th hour of incubation. With 3 washings it was estimated that the original culture fluid was diluted anywhere from 10,000 to 100,000 times. It may be noted from exper. 1, that after such dilution in 60% sucrose, or 20% NaCl, 2 c.c. were lethal to a guinea-pig in 42 hrs., while in exper. 3, the toxin titer of such dilutions was sufficient to kill the pigs in 30 and 36 hrs., respectively.

An analysis of the toxicity of the various wash waters is of interest. The organisms of exper. 1, 2, and 3, were from 24, 21, and 16-hour cultures, respectively, and the toxicity of the 3rd wash waters (the 2nd in case of exper. 2) was such that experimental guinea-pigs ingesting 2 c.c. quantities, lived, no symptoms; died in 39 hrs.; and died in 24 hrs., respectively. If the toxin titers are compared with the ages of the organisms washed, we note that the nontoxic wash water was from the 24-hour culture, while the most toxic was from the 16-hour culture, a fact which again argues against the possibility of having carried over the toxin with some of the original culture fluids. A hypothesis more in accord with the facts is as follows:

The toxin is an intermediary anabolic product not yet firmly bound in the cell, of which the young cells have a maximum amount. Sturges and Rettger²⁶ have recently expressed a belief fundamentally similar. It seems not impossible that the nonspecific toxic substances which Zinsser, Parker and Kuttner²⁷ secured in young bacterial cultures, particularly by washing young agar growths in salt solutions, may be substances from similar sources. The specificity of the toxin from the *B. botulinus* cultures could easily be due to a specificity in its anabolic and katabolic processes. It may be supposed that the younger, fragile cells are far more sensitive to osmotic effects than are the older, well

²⁶ Jour. Bacteriol., 1922, 7, p. 551.

²⁷ Trans. Soc. for Exper. Biol. & Med., 1921, 18, p. 49.

formed cells, and when they are removed from the culture fluids, rich in protective colloids, and suspended for washing in a watery solution even though it be one containing 0.8% NaCl, there is an outward flow of the toxin, resulting in a wash water of comparatively high toxicity. That osmotic processes do play a prominent part in this sudden release of toxin is further evidenced by the decidedly greater toxicity of the 3 sets of salt and sugar solutions 1 hr. after receiving the washed organisms than is shown by the distilled water suspensions. On the other hand, the 24-hour culture, it may be supposed, was composed of well formed individuals. The height of the reproductive period had undoubtedly been passed on or before the 18th hour, as has repeatedly been shown to be the case by plate counts of similar cultures in standard medium, and while the negative growth phase may have been initiated, the individual organisms are probably quite intact. In the ordinary toxin tests of bacterial cultures, this toxin reservoir is not tapped. The limited amount of this source of toxin is suggested by further data of exper. 3. The young organisms, which had so greatly enriched the 3rd wash water with toxin, after inoculation into the salt and sugar solutions evidently had little more toxin to lose, as all toxicity of the suspensions had been lost by the 7th day of incubation at 37 C. Dubovsky and Meyer¹⁶ have shown a distinct loss in toxicity of cultures in which some active autolytic action is in all probability still continuing during 10 days' incubation at 37 C. With the organisms in question the autolytic or usual source of toxin is probably greatly weakened by the lack of an extracellular proteolytic enzyme with the 16-hour organisms inoculated. Gelatinase production by *B. botulinus* cultures has been shown to occur only about the time that toxin can first be demonstrated. It is fully realized that autolysis may be due to a very different enzyme, a distinction which has been fully set forth for other organisms by Flexner²⁸ or to a group of enzymes as has been shown by Vines²⁹ for proteases of several plants, and by Dernby³⁰ for both yeast and tissue autolysis.

Plate counts and Hopkin's tube measurements have revealed a death as well as a disintegration or loss of mass in *B. botulinus* cultures. The reversibility of enzyme action outlined in detail by Bayliss³¹ accounts well for this suggested bimodal curve of toxin production. Bacterial autolysis has been studied recently by several workers, and the evidence

²⁸ Jour. Exper. Med., 1907, 9, p. 105.

²⁹ Ann. of Bot., 1904-1910, 18-24, Ser. 1 to 7, inclusive.

³⁰ Biochem. Ztschr., 1917, 81, p. 109; Jour. Biol. Chem., 1918, 35, p. 179.

³¹ The Nature of Enzyme Action, 1914, p. 49.

presented for other organisms bears a corroborative relationship to the present discussion. It should also be recalled that active proteolytic enzymes have been demonstrated in *B. botulinus* culture fluids. Sturges and Rettger²⁶ found proteolytic bacteria strikingly autolytic. Corper³² found that tubercle bacilli suspended in physiologic salt solution underwent autolysis at 37 C. with the liberation of an antigenic substance.

With respect to toxin production by spore inoculums into the 60% and 70% sugar and 20% NaCl containing-mediums, there are several phases of the question to be considered. Among these are probable effects of heating for 1 hr. at 80 C. on the vegetative forms, spore forms, toxins and enzymes. Without going into a discussion of the possibility of resistant vegetative forms, which has been touched on in preceding paragraphs, it may be asserted without fear of contradiction that wholesale death of these less resistant forms occurs. This leaves the most fertile substratums for autolytic enzyme action. Sturges and Rettger²⁶ found that most rapid autolysis of killed bacteria occurred within the first 24 hrs., while Corper³² found that the height of autolysis of tubercle bacilli occurred about the 7th or 8th day.

There is also no question but that the genuine endogenous spore forms are little affected by heating for 1 hr. at 80 C. There was only one instance in this series of experiments in which there was a possibility of any toxin remaining intact during the heating. Experimental evidence and a review of the literature by Schoenholz and Meyer on the destruction of toxin by heat is in process of publication. Without attempting a discussion of the question here, it may be stated that their conclusions negative completely the idea of the possibility of enough toxin remaining intact to account for any of the phenomena under discussion, as does the earlier work of Burke and of Orr.²⁰

Toxin production without growth has been demonstrated independently by other workers in this laboratory. J. Easton, in attempts to find a synthetic medium which will support growth and reproduction of *B. botulinus*, has repeatedly been able to demonstrate the presence of a comparatively potent toxin when no evidence of growth could be secured. Her inoculums were washed spores. Coleman and Meyer³³ found toxin produced not only by washed spores suspended in saline phosphate during a 48-hour stay in the icebox, but also in a second saline phosphate wash from the heated spores. The toxin-producing enzyme must have found in the wash water enough fragments of

³² Jour. Infect. Dis., 1916, 19, p. 315.

³³ Ibid., 1922, 31, p. 622.

bacterial bodies to furnish a substratum for its action. Thom, Edmondson and Giltner³ found a suspension of washed cells which had stood in the icebox over night toxic for rabbits and guinea-pigs. Flexner²⁸ found meningococci resisted autolysis longer at 37 C. than in the refrigerators. Evidence from the extensive enzyme literature suggests by analogy that heating for 1 hr. at 80 C. may have comparatively little effect on the enzymes of *B. botulinus*. Meyer³⁴ found the proteases of *Ps. pyocyaneus* little affected by 15 min. at 100 C., but no digestion took place during the heating; Wells and Corper³⁵ report that 30 min. at 100 C. does not suffice to destroy lipolytic bacterial enzymes, while Abbott and Gildersleeve's³⁶ experimental results also support the conclusion that 15-30 min. at 100 C. frequently does not destroy bacterial enzymes. In summarizing Effront's work, Waksman states:³⁷ "In the treatment of bacterial spores by antiseptics or by heat, it is found that the more difficult their germination the more productive they are of enzymes." This is suggestive, since the delayed germination of heated *B. botulinus* spores has been strikingly confirmed recently by Esty and Meyer,¹⁸ who report a maximum delay of 378 days. Not without interest is the conclusion of Kopeloff and Kopeloff³⁸ that cane sugar deterioration may be attributable to activity of enzymes of mold spores which have not germinated. Ruehle's³⁹ recent work is entirely in harmony.

That the toxin in *B. botulinus* cultures is a product emanating from bacterial protein is further suggested by the fact that the nature of the medium seems to exert little effect on the potency of the toxin produced, except as it hinders or promotes growth. This is especially true of the presence of glucose, which stimulates growth and increases toxin production.

There is considerable difference in the time of maximum toxin production in cultures incubated at 28 C. and at 37 C., if we may compare the statements in the literature. Burke²⁰ concluded that the optimum length of incubation at 28 C. is from 4 to 6 weeks, while Bengtson⁴⁰ and Dubovsky and Meyer¹⁶ set the maximum toxin production at 37 C. not far from 10 days. From a glance at the curves of growth for the two temperatures, shown in the preceding paper, it is

³⁴ *Biochem. Ztschr.*, 1911, 32, p. 388.

³⁵ *Jour. Infect. Dis.*, 1912, 11, p. 388.

³⁶ *Jour. Med. Res.*, 1903, 5, p. 42.

³⁷ *Abstr. of Bacteriol.*, 1922, 6, p. 265.

³⁸ *Jour. Agric. Res.*, 1919, 18, p. 209.

³⁹ *Abstr. of Bacteriol.*, 1923, 7, p. 7.

⁴⁰ *Am. Jour. Pub. Health*, 1921, 11, p. 352.

evident that no such difference exists in the rates of growth, but it is conceivable that the death and autolysis of the cultures might more nearly approximate this difference in the rate of toxin formation.

To summarize briefly: It seems not unlikely that toxin production in *B. botulinus* cultures is a function of enzyme action. The young growing cells are rich in the synthesized toxic substance which is readily given up under osmotic influences. Older cultures in which this preformed toxin is more closely bound in the cells liberate it only through cell autolysis. The enzyme or enzymes which probably are responsible for toxin production seem to be able to resist the heat which destroys the toxin.

CONCLUSIONS

No strain of *B. botulinus* cultured gave any evidence of growth in double strength veal infusion containing more than 8% of NaCl.

The maximum concentration of sucrose in which *B. botulinus* grows is approximately 50%. No proliferation was observed in 55% concentration.

Toxin production is not a reliable criterion of active proliferation of *B. botulinus*.

The evidence available seems to suggest that toxin production by *B. botulinus* is a function of enzyme action, and the bacterial cell is probably the matrix of the poison.

RESISTANCE OF SPORES OF *B. BOTULINUS* TO DISINFECTANTS. XVIII

CARRIE CASTLE DOZIER

*From the George Williams Hooper Foundation for Medical Research, University of
California Medical School, San Francisco*

*Aided by grants from the National Cannery Association, the Cannery League of California
and the California Olive Association*

Experimental findings relating to comparatively great resistance of living entities against destructive influences are of general biologic interest. The practical questions involved in the sterilization of hands, tables, incubators, and all laboratory utensils not adapted to prolonged treatment with moist heat are of utmost importance to any work involving sporulating organisms. The remarkable resistance to heat exhibited by spores of *B. botulinus* suggested that a comparatively equally high resistance to the action of ordinary laboratory disinfectants might be expected.

Since Koch's¹ pioneer work with spores of pure cultures of *B. anthracis* they have been the usually preferred indicator in testing the strength of disinfectants. Chick,² in fact, has produced from her results with anthrax spores and mercuric chloride, and from the results of Krönig and Paul³ and Madsen and Nyman,⁴ an ideal curve of disinfection. The abundant data relating to this organism cannot, of course, be applied to other species. Fortunately, before the investigation of the resistance of *B. botulinus* spores to disinfectants was undertaken, extensive work in this laboratory made it possible to approach the problem more nearly through the basic essentials for such tests so ably summarized by Krönig and Paul. One of their ideals was to have organisms of equal resistance as test material. With *B. botulinus* the problem was at first complicated by difficulty in securing consistent sporulation. Various theories as to the cause of bacterial sporulation have been current, and again the usual choice of organism for experimental work has been *B. anthracis*.

Received for publication, Jan. 30, 1924.

¹ *Gesammelte Werke von Robert Koch*, 1912, p. 287.

² *Jour. Hyg.*, 1908, 8, p. 92.

³ *Ztschr. f. Hyg. u. Infektionskr.*, 1897, 25, p. 1.

⁴ *Ibid.*, 1907, 57, p. 388.

CAUSES OF SPORULATION

Buchner⁵ thought the factor which contributed most to sporulation was exhaustion of food. Migula⁶ and Matzuschita⁷ recognized the importance of this but found other factors important, particularly the accumulation of metabolic products and the presence of oxygen, respectively. Schreiber⁸ was able to show that actively growing vegetative forms of *B. anthracis*, *B. subtilis*, and *B. tumescens* could be quickly transformed into spores if the growth was suddenly checked for any reason. One effective method of procedure was to centrifuge the cultures and resuspend the organisms in distilled water or a 2% sodium chloride solution, as Buchner had done. He did not believe a biologically poor medium ever favored sporulation. He refuted Buchner's "exhaustion theory" by resuspending vegetative forms from actively growing cultures in distilled water, a filtrate from a spore-rich culture and in the original culture fluid. The distilled water suspension sporulated first, while the filtrate suspension was the last to show spores. A close relationship of his views to those of Buchner, however, is suggested by one of his experiments. He cultivated 69 generations of *B. subtilis*, and 48 generations of *B. anthracis*, without sporulation, by regular renewal of the food. He concluded that sodium carbonate, magnesium sulphide, sodium chloride, and distilled water favored sporulation by their growth checking properties. The experiments of Weil,⁹ although he never so interpreted them, have been cited¹⁰ as proof of the fallacy of the "exhaustion theory." He secured a vigorous growth of anthrax bacilli in the filtrate from a sporulating culture reinoculated with a few of the spores which had just been removed. Gotschlich¹¹ states that Lehmann's and also Osborne's results showed the number of spores, as well as their ratio to vegetative forms, increased with the biologic value of the medium. The latter thought the nature of the strain of anthrax more important than the composition of the medium. Stephandis¹² found more spores in a biologically good medium than in one of low biologic value, but he states the difference was not great. Sporulation occurred earlier in the latter. Behring¹³ found that sporulation occurred if accumulation of metabolic products was not inhibitory, or inhibition

⁵ Centralbl. f. Bakteriöl., 1890, 8, p. 1.

⁶ System der Bakterien., 1897, 1, p. 173.

⁷ Arch. f. Hyg., 1902, 43, p. 267.

⁸ Centralbl. f. Bakteriöl., 1896, 20, pp. 353 and 429.

⁹ Arch. f. Hyg., 1889, 35, p. 355; Centralbl. f. Bakteriöl., I, Ref., 1903, 32, p. 257.

¹⁰ Klett: Ztschr. f. Hyg. u. Infektionskr., 1900, 35, p. 420.

¹¹ Handbuch. d. path. Mikroorganismen, 1912, 1, p. 144.

¹² Arch. f. Hyg., 1899, 35, p. 1.

¹³ Ztschr. f. Hyg. u. Infektionskr., 1899, 6, p. 117 and 7, p. 171.

was not caused by substances originally present, and the strain of anthrax was a sporulating one. He believed the process was a normal one and always occurred when not prevented in some way. Turro¹⁴ believed also that growth products interfered with sporulation. Koch,¹ in addition to appropriate food and a certain temperature, found oxygen necessary for the sporulation of *B. anthracis*. The lack of the latter accounted, in his opinion, for the inability of this organism to produce spores in the animal body. This same nonsporulation Buchner⁵ had ascribed to the nonexhaustion of food, and Behring,¹³ to the presence of carbon dioxide. Among others Turro,¹⁴ Kitasato,¹⁵ Buchner,⁵ Esmarch,¹⁶ and Behring¹³ also ascribed an important if not a chief rôle to oxygen, but the results of Klett¹⁰ and of Weil⁹ discredited this. The former, however, it should be noted, used paraffin oil as a means of air exclusion, and the anaerobic methods of the latter have been criticized. Jacobitz¹⁷ could get no sporulation in an atmosphere of pure nitrogen, nor could Shupski¹⁸ secure spores in the entire absence of oxygen, and his conclusions were confirmed by Gärtner¹⁹ and by Kuylentierma.²⁰ These results are not devoid of interest, since Matzuschita⁷ believed the presence of oxygen stimulated anaerobic sporulation.

Selter,²¹ using *B. oedematis maligni*, *B. anthracis symptomatici*, *B. tetani* and *B. botulinus* (?) concluded that the addition of 2% glucose or 5% glycerol favored sporulation, while Matzuschita⁷ found 10% of glucose optimum for the last named organism. Von Hibler²² found that sporulation of the anaerobes of his group 1 was much more dependent on the composition of the medium than was the case of group 2 (in which he included *B. botulinus*). The latter group sporulated abundantly, in their late stages of development particularly, in a medium of rich carbohydrate and small alkali content. In general, he believed an alkaline reaction the most important single factor favoring sporulation, while Matzuschita⁷ thought it only one of several important factors. Fitzgerald,²³ continuing the work of Noguchi²⁴ with organisms of the *aerogenes capsulatus* group, found that 1% of numer-

¹⁴ Centralbl. f. Bakteriöl., 1891, 10, p. 91 (abstract).

¹⁵ Ztschr. f. Hyg. u. Infektionskr., 1889, 7, p. 225.

¹⁶ Ibid., p. 1.

¹⁷ Centralbl. f. Bakteriöl., 1901, 30, p. 232.

¹⁸ Ibid., p. 396.

¹⁹ Cited from Sobernheim, Handbuch. d. path. Mikroorganismen, 1913, 3, p. 604.

²⁰ Ref. Centralbl. f. Bakteriöl., 1902, 34, p. 57 (abstract).

²¹ Centralbl. f. Bakteriöl., I. O., 1904, 37, pp. 186 and 381.

²² Untersuchungen über path. Anaeroben, 1908, p. 185.

²³ Jour. Path. & Bacteriol., 1911, 15, p. 147.

²⁴ Proc. N. Y. Path. Soc., 1907, 7, p. 196.

ous inert carbohydrates favored sporulation. The two most effective were mannite and amygdalin. The addition of fermentable carbohydrates acted as a deterrent to sporulation because of the resulting acidity. The presence of 0.5% to 5% of sodium chloride was neither stimulating nor inhibitive. This is not in harmony with the results of Buchner⁵ and Reichenbach²⁵ with *B. anthrax*.

Dickson, Burke and Ward²⁶ grew *B. botulinus* in sheep's brain medium for at least 2 weeks to produce spores for their heat resistance tests; Weiss²⁷ used spores which had grown in the same medium for approximately 1 month. He states that sporulation is rapid, and when complete approximately 15,000,000 spores per c.c. are present, from which it must be concluded that either growth was scant or that only a small fraction of the vegetative forms underwent sporulation. Burke²³ states that her strains did not readily sporulate in a brain medium. Hall²⁹ found anaerobic sporulation most abundant in mediums characterized by a low content of fermentable carbohydrates, such as deep brain, alkaline egg broth, meat infusion agar and blood agar slants. For *B. botulinus* heat resistance tests, he used 48-hour brain medium cultures. One outstanding characteristic of Hall's work is the variability in tendency to sporulation exhibited by the 5 strains of his collection. Esty and Meyer³⁰ found sporulation accelerated in certain mediums, but abundance of spores was not always an indication of the presence of highly resistant forms.

CAUSES OF VARIATION IN THE RESISTANCE OF SPORES

Regarding the causes of variations in the resistance of spores to heat and disinfectants, there have been widely divergent opinions. Before the era of pure cultures, Bucholtz,³¹ in 1875, sensed the important rôle of nutritional factors in the production of resistant organisms, for he wrote: "Bakterien, denen andere Nährstoffe das Material zu ihren Wachstum geliefert, mögen sich zu Antiseptica anders verhalten, mögen ihnen eine grössere oder geringere Resistenz entgehen setzen ich weiss es nicht, ich vermute es aber." ("Bacteria which obtained a supply of nourishment for their growth otherwise may behave differently in regard to antiseptics and may oppose more or less resistance to them.

²⁵ Ztschr. f. Hyg. u. Infektionskr., 1911, 69, p. 171.

²⁶ Arch. Int. Med., 1919, 24, p. 581.

²⁷ Jour. Infect. Dis., 1921, 28, p. 70.

²⁸ Jour. Am. Med. Assn., 1919, 72, p. 88.

²⁹ Jour. Infect. Dis., 1922, 30, p. 445.

³⁰ Ibid., 1922, 31, p. 650.

³¹ Arch. f. Exper. Path. u. Pharmacol., 1875, 4, p. 1.

I am not certain of this, I merely surmise it.") Ruzicka³² believed that anthrax developing on a one-sided food formed "sporoidkörper," differing from the usual spore forms in lessened resistance. Esmarch³³ showed by experimental work that both vegetative and spore forms differ in resistance according to their environment and the kind of food supplied them. Weil's⁹ spores grown at 37 C. were more heat resistant than those grown at 31, 24, or 18 C. Bellei³⁴ emphasized the desirability of using spores from abundantly sporulating cultures. Contrariwise, Zirolia³⁵ found that growth conditions had no effect on resistance, since he found all grades in the same culture. Geppert,³⁶ too, found individuals of very different resistance in anthrax spores prepared in the same way. Fraenkel³⁷ thought resistance a strain property, hence not affected by environmental factors.

Krönig and Paul³ subscribed to the heightened resistance of spores grown on food of high biologic value, but they also showed that the same food by no means always produced spores of equal resistance, nor could they greatly increase the resistance of anthrax spores by culturing through 10 successive mice, nor by growing from colonies developing from spores most resistant to HgCl₂. Von Hibler²² found the spores from a single species very constant in heat resistance if not too young, too old, too acid or too long exposed to incubator temperature. He found also that spores from brain, serum agar, potato and milk had approximately the same resistance. He concluded that shyly sporulating species in ill-adapted mediums produced spores which could scarcely be differentiated from granular forms of vegetative bacilli, and these appeared for short times only. Reiter³⁸ was successful in producing resistant anthrax spores on the wheat extract agar of Heider,³⁹ but concluded that many factors not under the worker's control also play important rôles in the production of resistant spores. Müller⁴⁰ also found this medium productive of resistant spores. Reichenbach²⁵ strongly recommends a Liebig's extract-peptone medium containing 2% of sodium chloride. It will be remembered that Buchner⁵ also found 2% sodium chloride effective in stimulating sporulation.

³² Arch. f. Hyg., 1908, 64, p. 219.

³³ Ztschr. f. Hyg. u. Infektionskr., 1888, 5, p. 67.

³⁴ München. Med. Wehnschr., 1904, 51, I, p. 301.

³⁵ Orig. not Available. Cited by Gotschlich, Handbuch d. path. Mikroorganismen, 1912, 1, p. 143.

³⁶ Berl. klin. Wehnschr., 1889, 26, pp. 789 and 819.

³⁷ Ztschr. f. Hyg. u. Infektionskr., 1889, 6, p. 521.

³⁸ Arch. f. Hyg., 1920, 89, p. 191.

³⁹ Ibid., 1892, 15, p. 341.

⁴⁰ Ibid., 1920, 89, p. 363.

In this laboratory it has been found that strains of *B. botulinus* differ markedly in their sporulating tendencies, but studies have revealed also certain general inclinations. Esty and Meyer³⁰ have summarized the results of studies based on growth in 24 different mediums, and in a previous section it has been shown that the addition of sodium chloride in amounts greater than the optimum for growth (approximately 0.5%⁴¹) did not stimulate sporulation, which is in accord with the results of Fitzgerald²³ with *B. aerogenes capsulatus*, but not in harmony with the results of Buchner⁵ and Reichenbach²⁵ with *B. anthracis*. Nor has glucose been found to stimulate sporulation, which is not in accord with Selter's²¹ and Matzuschita's⁷ results for *B. botulinus*.

With the exception of milk agar surface growths, the spores used throughout the experimental work on disinfectants were grown in 200-400 c.c. amounts of double strength veal infusion-peptic digest (equal parts) 10% gelatin, P_H 7.4, petrolatum stratified, and incubated at 37 C. This medium quite regularly produces 2 to 3 billion spores per c.c. in 48 to 96 hrs.

MEDIUM USED FOR GROWTH OF ORGANISMS SUBJECTED TO THE ACTION OF DISINFECTANTS

A second of Krönig and Paul's postulates which could be met because of preliminary work was that stipulating the use of a highly favorable medium for the recovery of the organisms after subjection to the action of disinfectants. Osborne⁴² had shown earlier that the number of anthrax spores germinating after such treatment depended on the biologic value of the culture fluid. Behring,¹³ for a reason which will be discussed in a subsequent paragraph, preferred a liquid medium for subcultures in preference to the solid medium used by Koch,¹ but Krönig and Paul³ considered a solid medium more reliable since it minimized the importance of survival of occasional individuals of high specific resistance. Chick,² Chick and Martin,⁴³ and Madsen and Nyman⁴ were among those who used solid mediums, while liquids were used by Anderson and McClintock,⁴⁴ Rideal and Walker⁴⁵ and the Lancet method.⁴⁶

⁴¹ Matzuschita,⁷ Tchitchkine (Ann. d. l'Inst. Pasteur., 1905, 19, p. 335), and Leuchs⁴³ (Ztschr. f. Hyg. u. Infektionskr., 1910, 65, p. 55) considered 0.5% salt most favorable for growth of *B. botulinus*, but Forsmann recommended 0.6% to 1% and van Ermengem's (Ztschr. f. Hyg. u. Infektionskr., 1897, 26, p. 1) (Cited from van Ermengem, Handbuch d. path. Mikroorganismen, 1913, 4, p. 909) original directions for pork infusion medium called for 1%.

⁴² Arch. f. Hyg., 1890, 11, p. 51.

⁴³ Jour. Hyg., 1908, 8, p. 654.

⁴⁴ Hygienic Lab. Bull., 82, 1912.

⁴⁵ Am. Jour. Pub. Health, 1913, 3, p. 575.

⁴⁶ Lancet, 1909, p. 1516.

Süpfle and Dengler⁴⁷ studied the effect of the biologic value of the medium used for growth of transplants and found the addition of 5% horse or cow serum to ordinary bouillon increased the time required for the destruction of anthrax spores from 80 min. to 160 min. in one test. Reiter's³⁸ more recent results are in harmony.

Preliminary work with *B. botulinus* has proved liquid mediums superior to any clear solid medium which can be used in test tubes in recovering small numbers of spores, and since absolute sterilization was the aim sought, Kronig and Paul's criticism would not apply. Peptic digest-beef heart mince-0.5% glucose has initiated growth and reproduction with the smallest number of organisms present. This medium Dubovsky and Meyer⁴⁸ found superior for the isolation of *B. botulinus* from food and field specimens. It was also the medium Esty and Meyer³⁰ found most favorable for the recovery of spores injured by heat, and has been used in this series of tests for all subcultures.

NUMBER OF SPORES USED IN TESTS

A third postulate of Krönig and Paul³ calls for the use of the same number of organisms in comparative tests. Dakin and Dunham⁴⁹ considered that some such number as 100,000,000 organisms per c.c. "will be found appropriate" in testing the strength of antiseptics. Lange⁵⁰ warns against the use of too large a number of organisms, and it may be noted that he found that dead bacteria are effective in raising the resistance against disinfectants. He thinks the employment of uniform quantities in a series of tests is more important than the actual number of organisms involved. Reichenbach⁵¹ has emphasized the fact that disinfection time depends not alone on the play of physical-chemical factors on the organisms, but also that the individual resistance of bacteria exerts a measurable effect. Ficker,⁵² moreover, has shown how results may be influenced by the smallest and apparently insignificant factor. Esty and Meyer³⁰ found that cultures of *B. botulinus* with less than one million spores may have a greater heat resistance than others containing several billion spores. Nevertheless, an attempt was made to control the number of organisms used in testing resistance against disinfectants by matching the turbidity of suspensions

⁴⁷ Arch. f. Hyg., 1916, 85, p. 189.

⁴⁸ Jour. Infect. Dis., 1922, 31, p. 501.

⁴⁹ A Handbook on Antiseptics, 1917, p. 77.

⁵⁰ Ztschr. f. Hyg. u. Infektionskr., 1922, 96, p. 92.

⁵¹ Centralbl. f. Bakteriöl., 1922, 89, p. 15.

⁵² Centralbl. f. Bakteriöl., 1920, 27, 685.

and by microscopic counts in a Helber chamber, and verifying these by the dilution culture method in both 0.5% glucose-peptic digest-beef heart mince and 0.5% glucose-peptic digest-beef heart infusion, P_H 7.2, petrolatum stratified after inoculation, and incubated at 37 C. for periods of at least 100 days.

EFFECT OF DISINFECTANTS CARRIED OVER ON GROWTH OF SUBCULTURES

A fourth Krönig and Paul postulate which received careful consideration concerns the carrying over of disinfectants. Geppert³⁶ first called attention to this probable source of error in Koch's work. By treating samples removed for cultivating with ammonium sulphide, he increased the time necessary to accomplish disinfection by 1.69% $HgCl_2$ from 12 min. to 1 hr. The spoons used in the Lancet method⁴⁶ carried over three times the amount of disinfectant that a standard loop does, and only 5 c.c. of subculture medium is called for, yet neutralization is not advised. Anderson and McClintock⁴⁴ and Rideal and Walker⁴⁵ found that 10 and 5 c.c. amounts, respectively, of broth avoided the antiseptic effect of disinfectant carried over, and a fluid subculture medium was recommended by Behring¹³ because of the better diffusion conditions afforded the traces of disinfectant. Woodhead⁵³ had earlier devised a method suitable for the use of either liquid or solid mediums but contrary to statements in the literature⁵⁴ no recommendation was made regarding the superiority of either. Emery⁵⁵ did not add a neutralizing agent even when using mercuric chloride and a solid medium. He advises the use of a large sized plate to permit space for diffusion of the disinfectant, which he does not believe is hindered by agar, a conclusion in which he is supported by Wells⁵⁶ and others. With phenol, Chick used a solid medium and concluded that the amount of disinfectant carried over in one drop samples was not sufficient to be inhibitory. Nevertheless, Hailer⁵⁷ believes that the carrying over of disinfectants is a serious objection to the several methods of testing disinfectants in which it is inherent.

Continuous observation has not shown that the small amount of disinfectant carried over into 10 c.c. amounts of medium with a loop 3 mm. in diameter has had a demonstrable antiseptic effect on *B. botulinus* spores. The explanation of this tolerance is probably to be

⁵³ Proc. Roy. Phys. Soc. Edinburgh, 1887, 9, p. 386.

⁵⁴ Blyth: The Analyst, 1906, 31, p. 150.

⁵⁵ Lancet, 1916, 1, p. 817.

⁵⁶ Chemical Pathology, 1920, p. 35.

⁵⁷ Deutsch. med. Wchnschr., 1921, 47, 2, p. 1384.

explained by various observations recorded in the literature and in observations made in this laboratory. Organisms vary greatly in their response to the antiseptic effect of mercuric ions and other disinfectants. Taylor⁵⁸ has discussed the selective action of antiseptics on various organisms, and Browning, Gilmour and Gulbranson⁵⁹ have made a bibliographic review. Anderson and McClintock⁴⁴ found the phenol coefficient varied as much as 300% when different organisms were used as test material, and Chick and Martin⁴³ found a variation of 1,000% under such circumstances.

Brooks'⁶⁰ results with 5 species of fungi spores indicated that the small percentage of the toxic agent carried over either served as a slight stimulus to germination and growth or exerted no appreciable influence. During the course of the experiments to be reported in subsequent paragraphs, it has been found that *B. botulinus* spores subjected to 0.02% iodine solution for periods up to and including 60 min. grew and reproduced more quickly than controls. Hotchkiss,⁶¹ in discussing results of studies on the stimulating and inhibitive effect of certain cations on bacterial growth, states: "In 15 of the 23 chlorides studied a concentration was found which stimulated growth." Not without interest is Pack's⁶² summary of the substances used to reduce dormancy and force seeds to germinate. He mentions concentrated sulphuric acid, sulphates, nitrates, hydrogen peroxide, ether, hot baths, alternating temperatures, etc. It may well be that the spores of *B. botulinus* which often lay dormant under apparently optimum conditions of growth for weeks and even months—in striking contrast to the quick germination of anthrax spores—are stimulated to development by substances inhibiting the latter. Crocker and Harrington⁶³ found HgCl_2 a good forcing agent for Johnson grass. Rose⁶⁴ advises that the effect of forcing agents is not so striking for quickly germinating seeds as where dormancy is involved. Schütze,⁶⁵ working with malignant edema spores, concluded that the sulphur compounds formed after neutralization with mercuric chloride acted as a slight stimulus to bacterial development, although Chick and Martin,⁴³ working with anthrax spores, while advising neutralization with a sulphur compound, add, "Such precipitants have themselves an inhibiting action upon the

⁵⁸ Lancet, 1917, 1, p. 294.

⁵⁹ Applied Bacteriology, 1918, p. 88.

⁶⁰ Bot. Gaz., 1906, 42, p. 359.

⁶¹ Jour. Bacteriol., 1923, 8, p. 141.

⁶² Bot. Gaz., 1921, 71-72, p. 32.

⁶³ Unpublished results cited by Pack (66, p. 39).

⁶⁴ Bot. Gaz., 1919, 67, p. 28.

⁶⁵ Brit. Med. Jour., 1915, 2, p. 921.

growth of bacteria, and it is important not to add enough to interfere with subsequent growth." It is not without significance, perhaps, that the spores used by Chick² were heated at 80 C. for 5 min. before subjection to the HgCl_2 , which may have increased the permeability of their outer membrane and made them more vulnerable to toxic effects of small traces of sulphur compounds. Bruyning⁶⁶ reported that a shower bath with boiling water so altered hard coated seeds that subsequent germination increased markedly. Crocker⁶⁷ has stated that the cause of delayed germination, characteristic of a great many seeds, generally lies in the seed coats which exclude water and oxygen necessary to growth, and anything which increases the permeability of the outer membrane without affecting adversely the contents increases and hastens germination. It is to be emphasized that the effect of any preliminary treatment of spores subsequently used to test the strength of disinfectants should be carefully investigated.

Another factor which should be mentioned in this connection is the observation first made by Behring¹³ that with optimum temperatures growth-inhibiting factors are more easily overcome. Brooks⁶⁹ observed the same phenomenon with fungi spores, and Chick² with paratyphoid organisms. It is likely that the optimum food conditions provided for *B. botulinus* by the peptic digest-beef heart mince-0.5% glucose medium helps materially to overcome any slight inhibiting action the small amount of disinfectant might otherwise exert.

METHOD OF TESTING DISINFECTANTS

The problem of testing the strength of disinfectants, or its corollary, the resistance of organisms to disinfectants, has been attacked by numerous investigators by several different "methods." These owe most of their specificity to the means employed for removing organisms from the disinfectant solutions and carrying them to the nutrient mediums. The pioneer work of Pasteur and Lister was followed by the investigations of Bucholtz,³¹ de la Croix⁶⁸ and others on the antiseptic strength of various substances added to mixed bacterial cultures. Koch,¹ using his carefully devised "thread" method, first scientifically attacked the problem of disinfection. His method immediately received severe criticism by Geppert,³⁶ but was defended quite ably by Behring.¹³ The chief criticism concerned the carrying over of small amounts of disin-

⁶⁶ Jour. f. Landwirtschaft, 1893, p. 86.

⁶⁷ Bot. Gaz., 1906, 42, p. 265.

⁶⁸ Arch. f. exper. Path. u. Pharmacol., 1881, 8, p. 175.

fectant into subcultures. Krönig and Paul's³ "garnet" method formed the basis for their most careful work, and Bechold and Ehrlich,⁶⁹ used agar surfaces, but this method never came into general use. Rideal and Walker's⁴⁵ "standard loop" method was devised in order to use vegetative forms of bacteria as test material, since only spores lend themselves to the former methods mentioned on account of the drying which is necessary. The sampling spoons required by the Lancet method⁴⁶ are not part of the equipment of the usual laboratory, and the loop has been preferred by most workers. Anderson and McClintock⁴⁴ used the loop, which in practice differs from the spoon in lessening the number of organisms subcultivated, and at the same time has the advantage of carrying over less of the disinfectant. Hailer⁵⁷ claims for his more recently devised "batiste" method the advantage of carrying over the maximum number of bacteria with the minimum amount of disinfectant of any of the "träger" methods, under which he included silk threads, glass rods and garnets. The "suspension" methods he believes even less desirable because of the small number of germs carried over in comparison with the large amounts of disinfectant. The cotton batiste pieces, he emphasizes, do not absorb so much of the disinfectant as wool or silk would, and at the same time they afford a greater surface for the germs to cling to. However, this method necessitates a drying procedure, which as will be shown later, lessens the resistance of *B. botulinus* spores.

The loop method was chosen as best adapted to the problem in mind, and two loops of No. 23 U. S. gage platinum wire 3 mm. in diameter were used in making the necessary transfers. They were sterilized by a fan-tail burner after the manner of Anderson and McClintock. A water bath packed in sawdust kept the disinfectant spore suspensions at a constant temperature of 20 C., and an electrically controlled bath was used for the tests made at 37 C. The spore suspensions and the disinfectant solutions were brought to the desired temperature before combining, and, since most of the experiments covered comparatively long periods of time, the test tubes which held the disinfectant-spore suspensions were plugged with sterile cotton stoppers.⁷⁰

⁶⁹ Ztschr. f. physiol. Chem., 1906, 47, p. 177.

⁷⁰ Disinfectants used: alcohol, 95% commercial; antiformin, American Antiformin Co.; chromic acid, crystals, C. P., J. T. Baker Chem. Co.; cresol, liquor cresolis compositus, guaranteed to contain 50% cresol and 50% linseed oil soap, Babrick Chem. Co.; formalin, 36.5% to 38.5% formaldehyde by weight; titration with N/10 sodium thiosulphate (Romijn method) showed 38% plus, of formaldehyde; Schering and Glotz, New York; hydrochloric acid, HCl 37.3%, sp. gr. 1.189, J. T. Baker Chem. Co.; iodine, resublimed crystals, dissolved in 0.6% potassium iodide aqueous solution, strength checked by titration with N/10 sodium thiosulphate, Mallinckrodt Chemical Works; lysol, Lehn and Fink, New York; mercuric chloride, J. T. Baker Chemical Co., C. P.; phenol, Mallinckrodt Chemical Works, acid carbollic, C. P.; trikresol, Schering and Glotz, New York.

INJURY AND RETARDED GERMINATION

Burke has recently expressed the opinion that retarded germination of heated spores is probably not an expression of injury.⁷¹ While no study of this phase of the problem has been attempted, the results of work with disinfectants are such as to favor the view that injury does result from their action. In any event, injury to the culture as a whole is plainly shown, and in such a sense the term is hereafter used. There is little evidence in the results herein reported which will support the view that impermeability of the spore wall is the chief determining factor in equipping the spore to resist the deleterious action of disinfectants and the growth-stimulating effect of nutrients in the subcultures. If such were the case accelerated germination might be expected of spores which had been subjected to disinfectants, or even heat, for lengths of time not lethal.

EFFECT OF DISINFECTANTS ON MOIST SPORES

Exper. 1: Five c.c. amounts of 5% phenol, 5% lysol, and 2% HgCl_2 were added to 0.5 c.c. of moist spore suspensions of strains 4 and 38 which had received no preliminary treatment except shaking with sterile sand to secure an even suspension. These preparations were kept at 20 C., and subcultures of 1 loop each were made hourly for 7 hours. The transplants from the mercuric chloride tubes after the 3rd hour exhibited a slight retardation of growth, suggesting slight injury. No retardation occurred in the cultures from the lysol and phenol tubes. Strain 4 spores subjected to 0.5% HgCl_2 , 2% phenol and 2% lysol suffered no injury during a 24-hour interval, during which a temperature of 20 C. was maintained during the first 9 hours, and then there was a slow drop to a minimum of 13 C.

Exper. 2: Five per cent. phenol, 5% lysol, 5% formaldehyde, 2% HgCl_2 and 0.5% trikresol were added in 5 c.c. amounts to 0.5 c.c. of strain 38 spore suspension treated as described in the preceding paragraph. The temperature was maintained at 20 C. during the 5 days of the experiment, but during the nights it fluctuated around 15 C. Frequent transplants failed to indicate any injury to the spores from 5% phenol, 5% lysol, or 0.5% trikresol. Retardation of growth in subcultures was evidence of some injury by 5% formaldehyde after 11 hours. The retardation increased consistently until at the end of 5 days the subculture failed to show growth until the 14th day of incubation. Transplants from the 2% HgCl_2 suspension showed slight retardation, but no increase of injury was indicated after the 11th hour.

EFFECT OF DISINFECTANTS ON DRIED SPORES

Strain 97 spores were centrifuged, washed twice with sterile 0.9% salt solution, mixed with sterile sand and dried in vacuo at 37C. over concentrated sulphuric acid. Esty and Meyer³⁰ found the heat resistance of spores treated in this way remarkably constant, although distinctly lessened. In order that comparative tests might be made on different days with spores of equal resistance, the procedure recommended by these workers was followed. Just before

⁷¹ Jour. Infect. Dis., 1923, 33, p. 274.

use the spores were resuspended in sterile salt solution and 0.5 c.c. amounts received 5 c.c. of the disinfectants.

The effect of temperature was also studied by preparing duplicate disinfectant-spore suspensions and holding one at 20 C. and the other at 37 C. As previously mentioned, the 20 C. bath was not constant, since the temperature decreased during the nights, but the 37 C. bath was electrically controlled, and therefore the temperature did not fluctuate during the experiment, which lasted 7 days. At 20 C., 5% phenol caused slight injury, which seemed not to increase after the 2nd hour. At 37 C., the retardation of growth quite consistently increased, until at the end of 7 days it took 33 days for growth to become evident in the subculture. Five % lysol retarded germination somewhat both

TABLE 1

ACTION OF DISINFECTANTS

Five-tenths c.c. dried spore suspension, strain 97, in 5 c.c. of disinfectant; 10 to 100 mm. spore per c.c. of spore suspension estimated by dilution count.

Time Subjected to Disinfectant	Disinfectant and Time in Days Growth First Appeared in Transplants 10 to 100 m. Spores Estimated in Transplants							
	5% Phenol		5% Lysol		5% Formaldehyde		2% HgCl	
	20 C.	37 C.	20 C.	37 C.	20 C.	37 C.	20 C.	37 C.
15 min.	2	4	2	3	2	13	6	6
30 min.	4	4	3	4	3	16	*	7
45 min.	3	4	4	7	4	62	4	—
60 min.	3	7	6	3	4	93	6	—
1½ hrs.	3	3	6	8	12	—	4	—
2 hrs.	6	7	3	8	11	—	6	—
3 hrs.	5	4	3	3	22	—	7	—
4 hrs.	6	6	4	5	19	—	16	—
5 hrs.	7	10	3	3	27	—	10	—
6 hrs.	4	11	4	3	27	—	13	—
7 hrs.	5	11	6	5	34	—	31	—
8 hrs.	3	5	3	3	43	—	15	—
22 hrs.	3	11	5	5	—	—	23†	—
28 hrs.	6	11	2	10	91	—	—	—
32 hrs.	5	16	5	5	—	—	—	—
48 hrs.	—	32	4	5	—	—	—	—
3 days	4	16	5	9	—	—	—	—
4 days	8	15	4	7	—	—	—	—
5 days	7	—	5	5	—	—	—	—
7 days	5	33†	4	4	—	—	—	—

* Broke.

† 2 c.c. orally to guinea-pig was fatal in less than 18 hrs

at 20 C. and at 37 C., but the injury sustained was not marked at either temperature. At 37 C., the injurious effect was less than that exerted by 5% phenol. At 20 C., 5% formaldehyde was lethal in 32 hours, while at 37 C., 1½ hours sufficed to prevent germination of transplants. Two % HgCl₂ at 20 C. caused death of all organisms in 1½ hours, and at 37 C., one-half of that time, 45 min., sufficed. Two c.c. amounts of the subcultures from the 7-day phenol and the 22-hour HgCl₂ tubes, 10 days after growth had appeared, were lethal to guinea-pigs within 18 hours when given orally. These data are listed in table 1.

Neither 95% or 77% alcohol was lethal in 7 days, nor was there evidence of greater injury by one than the other. Both slightly retarded growth in subcultures. One-tenth % iodine proved lethal in 15 min., both at 20 C. and at 37 C. Five % antiformin was lethal in 30 min. at both temperatures.

EFFECT OF PRESENCE OF 3% ORGANIC MATTER

Numerous investigators have found the bactericidal titer of disinfectants markedly weakened by the presence of organic matter. Walker and Summerville⁷² added starch; Fowler,⁷³ urine; Blyth,⁵⁴ milk; Chick and Martin,⁴³ dried feces; Emery,⁵⁵ reconstituted blood; and Anderson and McClintock,⁴⁴ a combination of peptone and gelatin in sufficient quantity to make 2% and 1%, respectively, in the spore-disinfectant mixture. The addition of the gelatin-peptone mixture seemed to

TABLE 2

ACTION OF DISINFECTANTS IN THE PRESENCE OF ADDED ORGANIC MATTER

Five-tenths c.c. dried spore suspension, strain 97, in 5 c.c. of disinfectant plus 3% organic matter (gelatin); estimated, 1 to 10 m. spores per c.c. of spore suspension.

Time Subjected to Disinfectant	Disinfectant and Time in Days Growth First Appeared in Transplants 1 to 10 m. Spores Estimated in Transplants							
	5% Phenol		5% Lysol		5% Formaldehyde		2% HgCl	
	20 C.	37 C.	20 C.	37 C.	20 C.	37 C.	20 C.	37 C.
15 min.	2	4	2	2	3	8	3	6
30 min.	3	5	2	2	3	24	4	4
45 min.	3	6	2	2	4	*	3	9
60 min.	5	7	3	4	4	82†	6	18
1½ hrs.	3	3	4	5	6	—	4	13
2 hrs.	4	6	4	2	6	—	8	22
3 hrs.	4	2	4	3	8	—	6	—
4 hrs.	17	6	3	3	14	—	8	—
5 hrs.	5	7	2	2	29	—	18	—
6 hrs.	6	6	5	3	22	—	4	—
7 hrs.	4	7	5	5	21	—	8	—
8 hrs.	8	5	3	9	36†	—	15#	—
22 hrs.	3	6	4	2	—	—	37	—
28 hrs.	7	9	3	4	—	—	—	—
32 hrs.	6	6	6	4	—	—	9	—
48 hrs.	4	7	5	6	—	—	—	—
3 days	3	5	6	5	135†	—	—	—
5 days	5	7	5	3	—	—	—	—
7 days	5	8	5	6	—	—	—	—

* Broke.

† Shake colonies typical.

2 c.c. orally to guinea-pig fatal in less than 18 hrs.

approximate more nearly ordinary laboratory conditions under which disinfection of pipets, tables, etc., is generally attempted, and the method of preparation of Anderson and McClintock was strictly adhered to. Dakin and Dunham⁴⁹ have stressed the necessity of adding the disinfectant solutions last, which was done in every case.

If the data listed in table 2 are compared with those in table 1, it may be noted that organic matter exerted little effect either on time of sterilization or length of retarded germination when the tests were carried out at a temperature of 20 C. At 37 C., the length of retarded

⁷² Cited in Lancet, 1909, p. 1516.⁷³ Ibid.

germination, after 7 days' treatment with 5% phenol, was decreased from 33 to 8 days by the organic matter, while the time necessary for the 2% HgCl_2 to be lethal was approximately quadrupled, increasing from 45 min. to 3 hrs. The higher temperature seemed to have little effect in increasing the potency of the 5% formaldehyde and 5% lysol in the presence of 3% organic matter. Transplants from the latter, both with and without organic matter, at the end of 90 min. failed to grow, while those from the former showed in each case only a moderately delayed germination.

COMPARISON OF RESISTANCE OF MOIST AND DRIED SPORES TO DISINFECTANTS AND TO HEAT

A spore suspension of strain 97 was washed once with sterile salt solution, and resuspended in distilled water. One-half of this suspension was centrifuged and the sediment mixed with an amount of distilled water equal to one-half of the original culture fluid, and tested immediately. The other half was centrifuged, and the sediment mixed with sterile sand and dried in vacuo over sulphuric acid for 24 hours, when an amount of distilled water equal to one-half of the original culture was added and an even suspension secured by shaking. Mixtures of equal parts of the suspensions and double strength disinfectants, making concentrations of 5% chromic acid, 0.15% iodine, 19% formaldehyde, and 10% HCl , were held at 37 C. for 1 hour, and subcultures made at 5-minute intervals. Neither 5% chromic acid, 0.15% iodine nor 19% formaldehyde were lethal to the moist spores in 1 hour, but they were lethal to the dried spores in 35, 45 and 50 min., respectively. Ten % HCl proved lethal to the moist spores in 35 min., and to the dried spores in 20 min., a difference which is hardly large enough to be significant. Spores from the same suspensions were heated by the Bigelow and Esty⁷⁴ method, used by Esty and Meyer in their extensive investigation of the heat resistance of *B. botulinus* and other anaerobic spores. Results show a toleration of 105 C. by the moist spores for 45 min. as against a possible 10 min. for the dried spores. No attempt is made to explain the growth secured in the 40-minute transplant from the dried spores when viability of intervening subcultures was not demonstrated.

The results as a whole attest to the destructive effect of drying, even under conditions which it was thought would effect little or no damage. These data are listed in table 3.

EFFORTS TO EFFECT STERILIZATION IN 60 MINUTES

Subcultures were made at 5-minute intervals. Not only was disinfection not accomplished at 20 C. within an hour by 0.5% iodine, 10% formaldehyde, 7% antiformin, 50% cresol, 50% lysol, or 1% chromic acid, but injury, as measured by retarded germination of transplants, was either not demonstrable or slight.

Neither 10% antiformin nor 15% formaldehyde accomplished disinfection within an hour at 37 C., but the results with the former were inconsistent. No

⁷⁴ Jour. Infect. Dis., 1920, 27, p. 602.

growth was secured in the 15, 20, 35, 40, and 50-minute subcultures. There was but little retardation in the remaining subcultures, including the 60-minute transplant. The results are undoubtedly explained by the heavy precipitate formed in the antiformin-spore mixture. This observation was confirmed during other tests. The 15% formaldehyde subcultures showed increased retardation of growth up to a maximum of 20 days for the 60-minute transplant, suggesting an approaching "end-point." Fifteen hundredths % iodine and 25% antiformin at 37 C. accomplished disinfection in 40 min., but in this connection attention is called to data listed in table 4.

TABLE 3

COMPARISON OF RESISTANCE OF MOIST AND DRIED SPORES TO DISINFECTANTS AND TO HEAT.
STRAIN 97 SPORES, WASHED ONCE WITH SALT SOLUTION

Dilution count, 10 to 100 mm. per c.c.; microscopic count, 625 mm. per c.c.

Time Subjected to Disinfectant or Heat at 105 C.	Time Growth Appeared in Transplants									
	37 C.						20 C.		Heat, 105 C.	
	5% Chromic Acid		0.15% Iodine		20% Formal- dehyde		16% HCl		2 c.c. Trans- plants	
	Moist	Dried	Moist	Dried	Moist	Dried	Moist	Dried	Moist	Dried
	Days	Days	Days	Days	Days	Days	Days	Days	Days	Days
5 min.	2	2	2	2	2	3	3	3	*	4
10 min.	2	—	2	3	3	4	2	2	*	6
15 min.	—	—	2	3	5	6	2	1	2	—
20 min.	2	3	2	6	7	9	2	2	2	—
25 min.	3	18	3	8	9	10	2	2	*	—
30 min.	—	3	2	21	11	12	2	—	3	—
35 min.	2	—	4	—	15	21	—	—	3	—
40 min.	4	—	6	9	3	15	—	—	4	6†
45 min.	2	—	5	—	6	20	—	—	9	—
50 min.	7	—	5	—	9	—	—	2	—	—
55 min.	2	—	9	2	9	—	—	—	*	—
60 min.	5	—	6	—	15	—	—	—	—	—

* No test made.

† 2 c.c. given orally to 800 gm. guinea-pig; death in less than 24 hrs.

EFFECT OF DILUTION OF SPORE SUSPENSION ON THE LETHAL ACTION OF IODINE AT 20 C. AND AT 37 C.

One-tenth % iodine failed to exert any deleterious action in 1 hour on 50 to 500 million spores per c.c. on a dilution of 1:10, or on a dilution of 1:100, at 20 C. At 37 C., 0.16% and 0.08% iodine was all but innocuous to the undiluted spore suspension and to the 1:10 dilution. However, the 0.1% iodine began to have a marked effect on the undiluted suspension after 40 min. as evidenced by a retardation of growth of 9 days, which by the end of 60 min. had increased to 19 days. The 1:10 dilution subculture was no longer viable after 50 min.

COMPARISON OF RESISTANCE OF DRIED SPORES IMMEDIATELY AFTER RESUSPENSION AND AFTER STANDING 4 HOURS AT ROOM TEMPERATURE

In view of the observation by Fischöder⁷⁵ that anthrax spores suspended in distilled water germinate rapidly, it seemed of interest to investigate the effect,

⁷⁵ Centralbl. f. Bakteriöl., I, O., 1909, 51, p. 320.

as measured by changes in resistance to disinfectants, of allowing a suspension of *B. botulinus* dried spores to stand at room temperature for 4 hours. In no case was there any demonstrable loss of resistance. Comparative tests showed that 5% chromic acid and 0.6% iodine at 20 C. for 1 hour were quite without effect on dried spores just resuspended and on those which had stood at room temperature for 4 hours after resuspension. Both sets of chromic acid treated spores exhibited a slight increase in the length of the lag period over that usually obtained with dried spores. The 10% HCl was lethal in 40 and 45 min., respectively, which for practical purposes may be considered equal. These data are listed in table 4.

RESISTANCE OF SPORES GROWN ON THE SURFACE OF MILK AGAR

A 48-hour surface growth of strain 62 on glucosc-liver agar, to which approximately 10% of sterile milk had been added just prior to pouring the plate, was washed off with distilled water and thoroughly emulsified. Three

TABLE 4
COMPARISON OF RESISTANCE TO DISINFECTANTS AT 20 C. OF ONCE WASHED AND DRIED SPORES, STRAIN 97, IMMEDIATELY AFTER RESUSPENDING AND AFTER STANDING 4 HRS. AT ROOM TEMPERATURE

100 to 1,000 m. spores per c.c. of spore suspension estimated by dilution count; figures denote time growth first observed in transplants.

Time Subjected to Disinfectant, Min.	10% HCl		5% Chromic Acid		0.6% Iodine	
	Immedi- ately	4 Hours Later	Immedi- ately	4 Hours Later	Immedi- ately	4 Hours Later
5	2	2	2	2	2	2
10	2	2	2	2	2	2
15	3	2	2	2	2	2
20	5	18	2	2	2	2
25	3	13	2	2	2	2
30	—	10	3	2	2	2
35	3	—	3	3	2	2
40	—	3	3	3	2	2
45	—	—	3	3	2	2
50	—	—	3	3	2	2
55	—	—	3	3	2	2
60	—	—	5	3	2	2

c.c. amounts of this suspension were combined with 20% chromic acid, 38% formaldehyde, 37.3% HCl, 4.32% iodine, and sodium hypochlorite of 0.45-0.50% NaClO strength. The HCl and sodium hypochlorine suspensions were made in duplicate, and one set was incubated at 20 C. The remaining tests were made at 37 C. The spore suspension was the most dilute of any used during the course of the experiments, and undoubtedly accounts for the slightly lower resistance indicated. If the length of survival in the 10% HCl at 20 C. is compared with that of the dried spores of table 4, we note a survival of the latter for 40 min., while the milk agar surface spores withstood 10% HCl for only 20 min. However, even in 10% chromic acid at 37 C. growth of the milk-agar spores occurred after 10 min., while viability was preserved after 30 min. in 19% formaldehyde at 37 C. The dried spores had withstood that strength of formaldehyde for 45 min. and moist ones were still viable after 60 min., although distinct retardation of growth in transplants was evident.

DISCUSSION

The moist spores used in the tests listed in table 3 exhibited the greatest amount of resistance to disinfectants of any used during this series of experiments, and yet their resistance to heat did not reach the maximum reported by Esty and Meyer (45 min. at 105 C. as against their 85 min. at the same temperature, with an average survival time of 35.2 min.). At 37 C., 5% chromic acid, 0.15% iodine, and 19% formaldehyde all failed to effect sterilization within an hour. Judging from the length of delayed germination in subcultures, the 19% formaldehyde solution was most destructive to the moist spores; but to the dried spores, the 5% chromic acid was most destructive. Ten % HCl is practically as potent against moist as dried spores. The results do not confirm for *B. botulinus* the increased resistance which Krönig and Paul³ found with dried anthrax spores. They do confirm Esty and Meyer's³⁰ results with *B. botulinus*, which showed a loss of heat resistance through drying. Bellei³⁴ reported results showing that dried anthrax spores failed to survive after 6 hrs. of treatment with 0.1% HgCl₂, while moist organisms from the same culture withstood 2% HgCl₂ for 7 hrs. Dried spores are to be recommended for comparative tests where test material of equal resistance is desirable, but for testing the strength of disinfectants, or the resistance of organisms against destructive influences, moist spores should be used.

Madsen and Nyman⁴ found the velocity of disinfection of anthrax spores by mercuric chloride increased two and one-half times for a rise in temperature of 10 C. between 25 C. and 45 C., but Chick² found the temperature coefficient of disinfection varied from 2 to 10 according to the disinfectant and the species, age and number of bacteria used. While the data secured are not such as to make mathematical comparisons possible, a confirmation of Chick's conclusion, "That there is a very great advantage in the use of warm solutions for practical disinfection" is fully warranted. For instance, at 20 C., 5% formaldehyde accomplished disinfection in 32 hrs., and at 37 C. in 1½ hrs.; 2% HgCl₂ destroyed the viability of spores in 28 hrs. at 20 C., as against 45 min. at 37 C.

The accelerating influence of temperature on the death of *B. botulinus* spores is little changed by the presence of 3% organic matter when formaldehyde is the disinfectant used, but the potency of mercuric chloride is measurably lessened (tables 1 and 2). An isolated delayed germination period of 138 days after 3 days' exposure at 37 C. to

5% formaldehyde with the organic matter present is the only suggestion of a protective action of such addition. Bellei³⁴ accounted for similar results with anthrax spores by postulating the existence of occasional organisms endowed with a greatly heightened resistance. Dakin and Dunham⁴⁹ seem inclined to give credit for such results to the protective action of organic matter, citing as an example the presence of a few viable *Staphylococcus aureus* organisms after subjection for 3 hrs. to mercuric chloride, when at the end of the first 5 min. there had been a 90% destruction.

At 37 C., in the presence of 3% organic matter, the time necessary for 2% HgCl_2 to be lethal was approximately quadrupled over that required at 20 C., increasing from 45 min. to 3 hrs. The presence of 3% organic matter in the 5% lysol tubes appeared to have little effect, but an end point was not approached. It may be mentioned that Schottelius⁷⁶ reported no difference in the disinfecting power of lysol on *B. typhosus* in serum, bouillon or water, at room temperature. Brown-ing⁵⁹ states that serum does not reduce the potency of phenol, and he cites as unique the increase of potency of diamino-acridine (proflavine) and its derivatives in the presence of serum. On the contrary, Chick and Martin⁴³ have never failed to find a lessening of destructive action of disinfectants in the presence of organic matter, although the protective action varied with the organic matter and the disinfectant used. The reasons for the different degrees of interference have been discussed more or less in detail by Chick and Martin, and more recently by Traube and Somogyi.⁷⁷

Among the substances tried for disinfecting power, alcohol was without effect, either undiluted or diluted. This is in accordance with results with other organisms recorded in the literature; in fact, Koch¹ before subcultivating, washed anthrax spores with alcohol to free them from mercuric chloride. Five-tenths % trikresol was without effect even after 5 days, as was true of 5% phenol and 5% lysol at 20 C. when moist spores were employed. The rapid appearance of growth in transplants from these disinfectant-spore suspensions even suggests a stimulating action. With dried spores, 5% phenol and 5% lysol caused delayed germination, both at 20 C. and at 37 C. after times varying from a few hours to 7 days. Little deleterious effect was exerted by 50% lysol or 50% cresol in 1 hr. at 20 C., even on spores whose resistance had been lessened by drying. These results do not confirm the greater

⁷⁶ München. med. Wehnschr., 1890, 37, pp. 335 and 357.

⁷⁷ Biochem. Ztschr., 1921, 120, p. 90.

disinfecting power of the higher homologues of phenol found by Henle⁷⁸ and by Fraenkel,³⁷ with anthrax spores. Nor is there any evidence that the sensitiveness of *B. botulinus* spores to 5% phenol at higher temperatures would approximate that of anthrax spores at 33 C., disinfection of which Chick² found to be nearing completion after 7-8 hrs., and Behring,¹³ complete disinfection after 3 hrs. at 37.5 C. Results, however, tend to confirm the numerous conclusions as to the weakness of phenol as a disinfectant. Bellei³⁴ found anthrax spores treated with 5% phenol alive after 34 days; Esmarch,³³ 40 days; Guttman and Merke,⁷⁹ 37 days. Schütze⁶⁵ found 5% phenol without lethal effect on malignant edema spores after 8 days, but, on the other hand, Kitasato¹⁵ found spores of *B. tetani* without viability after treatment for 15 hrs.

B. botulinus spores offered only a moderate resistance to mercuric chloride. Five-tenths % at 20 C. for 24 hrs. did not cause delayed germination in subcultures; nor did 2% solutions at the end of 5 days cause more than a few hours delay in the appearance of growth. Its destructive effect was much accelerated at 37 C., but was adversely affected by the presence of 3% organic matter. Krönig and Paul³ found that 1.69% solution killed all anthrax spores in 12 min. at 20 C. In the light of experimental findings reported herein, a warning must be sounded against Chick and Martin's⁴³ conclusion, "In the case of spores metallic salts are the most efficient germicides." Unconditional acceptance would lead to false security.

Antiformin, unless used in almost full strength, shows little potency in the destruction of *B. botulinus* spores. Its tendency to cause a voluminous flaky precipitate in spore suspensions does not recommend it as an efficient germicide for *B. botulinus*.

Sodium hypochlorite is ineffective. The viability of spores of *B. botulinus* in a 50% solution (0.45-0.50% NaClO strength diluted once) for periods approximating 1 hr. at 20 C. and at 37 C. suffered little. These findings were unexpected in view of the rapid dissolving action attributed to hypochlorite solutions. The explanation probably lies in its tendency to rapid decomposition and consequent loss of all destructive power.

Ten % HCl failed in no case to effect sterilization within an hour. The continuous destructive effect of formaldehyde solutions is striking. A 5% solution at 20 C. exerted an apparently continuous deleteri-

⁷⁸ Arch. f. Hyg., 1889, 9, p. 188.

⁷⁹ Virchow's Arch. f. path. Anat., 1887, 107, p. 459.

ous action for 5 days, during which time a 2% HgCl_2 solution was inert after the 11th hour. Another point in favor of formaldehyde is the lack of depression of destructive power by the presence of 3% organic matter. Warm solutions are much increased in effectiveness. It may well be that formaldehyde has a selective action on *B. botulinus* spores. Slater and Rideal⁸⁰ found its effect differed according to the species of organism tested. Krönig and Paul³ report death of all anthrax spores in a 35% solution at 20 C. some time between 10 and 60 min., while Pottevin⁸¹ secured sterilization in 30 min. at 35 C. Van Ermengem and Sugg⁸² found that anthrax spores which survived 5% phenol for 6 days were killed by 40% formaldehyde in 3-12 hrs.

SUMMARY AND CONCLUSIONS

Recommendations relating to the chemical sterilization of laboratory utensils contaminated with *B. botulinus* are as follows: 10% HCl at room temperature may be depended on to destroy all *B. botulinus* spores within an hour. If a longer time can be allowed for sterilization, commercial formalin diluted once with warm water acting over a period of at least 24 hrs. is recommended. It is quite probable that such a solution held at 60 to 70 C. for a shorter time would be as efficient.

The advantages, in testing the strength of disinfectants, of using spores which respond to the effects of disinfectants in dilutions or for lengths of time not lethal, in a way that may be measured, are obvious. With a great many other species, e. g. spores of *B. anthracis*, results which are reported in the literature are either positive or negative. *B. botulinus* spores offer a finer measure of the effects of dilution, temperature, addition of organic matter, etc., through the lengths of the retarded germinations.

⁸⁰ Lancet, 1894, p. 1004.

⁸¹ Ann. d. l'Inst. Pasteur, 1894, 8, p. 796.

⁸² Centralbl. f. Bakteriöl., 1896, 19, p. 91 (abstract).

BACTERIOLOGIC STUDY OF MIDDLE EAR INFECTIONS

EUGENIA VALENTINE

From the George Williams Hooper Foundation for Medical Research, University of California Medical School, San Francisco

This study was aided by grants from the John C. and Edward Coleman Memorial Fund

This investigation of acute and chronic otitis media was approached from a different standpoint than any previously reported. Although interested in the relative prevalence of the different organisms, the primary object was to determine, if possible, factors contributing to chronicity and impaired hearing. It seemed that by studying a certain number of individual cases over a long period of time, with a close correlation of the clinical and bacteriologic findings, there might be some indication of the factors involved. Furthermore, the source of the ear infection was another point under investigation.

PLAN AND ORGANIZATION

The following points were considered:

1. The relative prevalence of the different bacterial varieties in a series of acute and chronic cases of otitis media.
2. The bacterial flora of the discharge of the ear of a certain number of patients with acute infections at intervals extending over a considerable period of time. It was anticipated that the findings might throw light on the course and the prognosis of the infection.
3. The throat flora of a number of persons with acute respiratory infections prior to the development of an ear condition in order to trace bacteriologically the source of the ear infection.
4. The throat condition in all cases of otitis media at the time of the original paracentesis and also later to determine whether the anatomic, clinical, and bacteriologic picture of the throat had any relation to the severity and subsequent history of the ear condition.
5. A detailed study of the bacterial variety found in the majority of the acute cases in order to ascertain whether there was a specific serologic variety which had a peculiar affinity for an elective localization in the ear.

6. A consideration of the ultimate condition of chronicity or deafness in the cases of acute middle ear disease from the standpoint of the original bacterium found in the ear infection.

7. The relation of the different varieties of secondary invaders to the final condition of chronicity.

LITERATURE

The bacterial varieties found in acute and chronic otitis media have been the subject of study for many years. A review of the literature shows that there is a divergence of opinion in regard to the organisms which occur most frequently. This is not surprising, since the predominance of certain varieties and their virulence may vary from time to time. The relative frequency of the different organisms found in acute middle ear infections as reported by various workers is as follows Reik,¹ pneumococcus, streptococcus, staphylococcus; Phillips,² pneumococcus, *Streptococcus pyogenes*, staphylococcus, Friedländer bacilli and diphtheroid bacilli; Albert,³ studying 110 cases found streptococcus in 100, staphylococcus in 5, pneumococcus in 3 and saprophytes in 2. Neumann and Ruttin,⁴ using a sterile capillary tube to which was attached a Politzer bag for suction, gathered their ear cultures in 97 cases through a perforation if one existed, and if not the capillary tube was used for drum puncture. The latter procedure is not to be recommended as it is a puncture and not an incision. Their findings were *Streptococcus pyogenes* 34, with 20 additional cases in which *Streptococcus pyogenes* was mixed with other organisms, such as staphylococcus, diphtheroid bacillus, *B. proteus* and gram-negative cocci; staphylococcus 11, *Streptococcus mucosus* 18, *Diplococcus pneumoniae* 2 and *B. pyocyaneus* 3. Kümmel,⁵ obtaining cultures by means of a fine pipet, reports the predominating organisms in 30 cases as follows: *Streptococcus pyogenes* 18, *Streptococcus mucosus* 3, *B. lancolatus* 4, *Staphylococcus aureus* 2, sterile 3. Armstrong⁶ concludes that 90% of acute cases are caused by the beta hemolytic streptococcus of the *pyogenes* group.

In chronic conditions of the middle ear, the bacteria most frequently found, according to published reports, are the diphtheroid bacillus, *B. proteus*, staphylococcus, *B. pyocyaneus* and *B. coli*.

Owing to the more recent advances in the differentiation of bacteria by biologic and serologic methods, much of the data in the older literature are inadequate in identifying the exact subgroups encountered.

COLLECTION OF MATERIAL AND BACTERIOLOGIC METHODS

Material from the ear discharge was obtained in the outpatient clinic by washing the paracentesis knife, previously sterilized in alcohol, in a tube containing 1 c.c. of beef broth, or if perforation had occurred, by taking through a sterile speculum some of the discharge from the site of the perforation by means of a fine applicator or a cotton swab on a copper wire. In chronic cases, the pus was taken directly from the middle ear whenever the size of the per-

¹ Jour. Am. Med. Assn., 1907, 49, p. 681.

² Arch. Otol., 1903, 32, p. 1.

³ Arch. f. Ohrenheilk., 1911, 85, p. 251.

⁴ Arch. f. Ohrenheilk., 1909, 79, p. 1.

⁵ Verhandl. d. deutsch. otol. Gesellsch., 1906, p. 227.

⁶ Iowa State Med. Soc., 1920, 20, p. 209.

foration permitted. Nasopharynx cultures were taken as high up and laterally as possible through the mouth by means of sterile swabs which were placed immediately in the tubes of broth.

BACTERIOLOGIC METHODS

Broth tubes which had been inoculated with the ear discharge or with the material on the throat swab were brought to the laboratory within an hour after collection. Direct smears were made and stained by the Gram method, and a large loopful of the material was streaked on the surface of standard (1 c.c. defibrinated sheep blood and 12 c.c. agar) blood-agar plates in duplicate, one for aerobic and the other for anaerobic cultivation. Pour blood-agar plates were made also in order to differentiate the varieties of streptococci more accurately. The aerobic plates were examined after 24 hours' incubation at 37 C., but isolations from the colonies were not made until the end of 48 hours. The plates for anaerobic growth were incubated for 48 hours in a jar from which the air had been exhausted and replaced by hydrogen. The original broth tubes, to which a drop of sterile defibrinated blood was added, were incubated for 24 hours and used for plating in the few instances in which the direct plates from the material showed little or no growth. Quantitative methods were attempted but were discontinued because the amount of material on the paracentesis knife or the swab of the ear discharge was not constant, and because 24-hour enrichment cultures were used occasionally. However, the relative number of organisms per field and the proportion of the different varieties in the direct smear were noted. After the first 25 cases had been examined, the anaerobic method was discontinued as the results did not warrant its continued use. Many of the organisms, such as the beta hemolytic streptococcus were facultative anaerobes, but no varieties were observed on the anaerobic plates, which were not present also on those subjected to aerobic conditions.

NOMENCLATURE OF THE STREPTOCOCCI

The nomenclature of the streptococci used in this study is based on the work of Smith and Brown⁷ and Brown,⁸ and is so familiar to workers in this field that the exact definition of the different types seems unnecessary. The beta hemolytic streptococcus is used to characterize the true hemolytic streptococcus. The alpha or green-producing streptococcus refers to the large group of gram-positive cocci, which produce varying amounts of green coloration when grown on the surface of blood-agar plates and which also, when poured in the plates, showed microscopically some red blood cells intact about the colony, surrounded by a secondary zone of clearing (alpha hemolysis) which varies considerably in width and clearness.

The term green streptococcus is adhered to in this work due to the confusion in certain publications, in which the alpha streptococci are tabulated as true hemolytic streptococci. Brown⁸ states that all green-producing streptococci belong to the alpha type.

The gamma or indifferent streptococci are small colorless colonies on the surface of blood-agar plates and produce no effect on the red blood cells when poured in the plate. No alpha prime (see Brown) colonies were encountered in this work.

⁷ Jour. Med. Res., 1914-1915, 31, p. 455.

⁸ Monograph Rockefeller Inst., 1919, No. 9.

RESULTS

The results of the primary examination of 100 acute infections of otitis media is given in table 1.

The specimens which were obtained from the original paracentesis yielded only one variety of bacteria in the majority of instances, whereas material from ears which had been draining for a period of

TABLE 1
BACTERIAL VARIETIES IN ACUTE EAR INFECTIONS BASED ON A STUDY OF THE PRIMARY DISCHARGE FROM 100 INFECTED EARS IN 77 CASES

Bacteria	No. of Infected Ears	%
Beta hemolytic streptococcus*	50	53
Beta hemolytic streptococcus and staphylococcus	3	
Total Beta hemolytic streptococci	...	
Pneumococcus type 3	1	
Pneumococcus group 4	7	9
Pneumococcus group 4 and green (alpha) streptococci	1	
Total pneumococcus	...	
Green (alpha) streptococcus	5	
Staphylococcus and green (alpha) streptococcus	3	22
Staphylococcus	22	
Diphtheroid bacilli	3	
Diphtheroid bacilli and staphylococcus	3	
Sterile	2	
Total	100	

* In the succeeding table the abbreviations used are as follows: B. hem. strep. indicates beta hemolytic streptococcus; green strep., green (alpha) streptococcus; staph., staphylococcus; B. or bac., bacillus; diph., diphtheroid.

TABLE 2
BACTERIAL VARIETIES IN THE DISCHARGE FROM 12 CHRONIC EAR INFECTIONS

Bacteria	No. of Cases
B. proteus	1
B. proteus, fusiform bacilli and spirilli	1
B. pyocyaneus	1
Diphtheroid bacilli	2
Diphtheroid bacilli and few colonies of B. hem. strep.	1
Staphylococcus 1 albus, 1 aureus	2
Staphylococcus aureus and few colonies B. hem. strep.	1
Staphylococcus and diphtheroid bacilli (equal proportion)	1
B. coli	2
Total	12

several days or more usually contained several varieties. The beta hemolytic streptococcus was found often in pure culture at the first paracentesis, and the same ear when cultured a week later contained staphylococci or diphtheroid bacilli mixed with the beta hemolytic streptococcus. In table 1, therefore, the organisms associated with the beta hemolytic streptococcus are disregarded, except in cases in which the proportion of beta hemolytic streptococcus is very small.

Twelve chronic cases were examined, with results as shown in table 2. The organisms encountered are those which others have found associated with chronic ear discharges. In one case the diphtheroid bacillus was morphologically so similar to the true Klebs-Loeffler bacilli that a virulence test was carried out. The culture proved to be nonvirulent.

CORRELATION OF EAR AND THROAT FLORA

As previously stated, the throat flora in the cases of otitis media was studied in the hope that the correlation of the bacteria found in the throat and in the ear discharge might yield information concerning the source of the ear infection. Shambaugh⁹ states that "acute otitis media is rarely encountered as a primary disease but as secondary to acute inflammatory reaction involving the membranes of the nose and throat." If a study of the throat flora should indicate that acute ear conditions are more frequent successors to respiratory conditions in which one variety of bacterium predominates, one might approach ear infections from the preventive standpoint.

Although the histories of middle ear infections show that the majority are sequelae of an acute cold or sore throat, no data have been presented in the literature to prove that the organisms which are present in the infection of the nose or throat are similar to those found in the ear discharge a few days later. In order to verify this point, the necessity of obtaining throat cultures before an ear condition has developed is obvious. A number of persons with inflamed throats have therefore had cultures made, but no ear conditions followed. On the other hand, the results of the bacteriologic examination of the throat taken simultaneously with the primary ear culture, although inadequate as a final proof of the source of the ear infection, may be of certain value, especially when the throat condition is still acute at the time cultures are taken.

Before attempting a discussion of the throat flora in relation to the organisms found in the ear discharge, the throat flora of normal persons must be considered in order to have a satisfactory basis of comparison.

The significance of the types of bacteria, especially of the beta hemolytic streptococcus found in the throat normally and during disease, has been considered in numerous publications. A review of these reports shows a divergence of opinion in regard to the presence of the beta hemolytic streptococcus in the throats of normal persons, depending to a certain extent on the methods employed. The high percentage of beta hemolytic streptococcus in the throat of

⁹ Illinois Med. Jour., 1922, 42, p. 431.

healthy persons during 1918-1919, as reported by Nichols and Bryan,¹⁰ Levy and Alexander,¹¹ Walker,¹² and others, would seem to indicate that an unusual carrier state existed at that time, coincident with the epidemics of influenza, measles and scarlet fever. In 1919 Pilot and Davis¹³ reported that the tonsils were the important breeding places for beta hemolytic streptococci; they showed that this bacterium was present in 95% of excised tonsils (majority hyperplastic) when cultures were taken from the crypts, 61% from the tonsil surface and 43% from the nasopharynx of the same persons before tonsillectomy. Tongs¹⁴ showed that the throats of persons whose tonsils had been removed yielded hemolytic streptococcus less frequently than those with tonsils. Bloomfield¹⁵ concludes that the normal throat flora consists of nonhemolytic streptococcus (gray or green), gram-negative cocci and influenza bacilli, with other organisms such as pneumococcus group 4, diphtheroid bacilli and staphylococcus as occasional transients. The beta hemolytic streptococcus in his opinion is not a normal inhabitant of the surface of the upper respiratory tract, but its presence indicates either a focus of infection, or a transient carrier state from a previous infection or from contact. In a more recent publication, Bloomfield and Felty¹⁶ have reported the results of a study of 108 healthy persons made at a time when streptococcus infection was not prevalent. In this series, the material was aspirated from the tonsil crypts by suction. The results showed that 9.5% of persons without tonsils harbored the beta hemolytic streptococcus in small numbers in the throat, whereas 41% of persons whose tonsils were intact were carriers of this organism in the crypts. They confirmed the work of Pilot and Davis¹³ in that they frequently found beta hemolytic streptococcus in the tonsillar tissues of persons from whom a pharynx culture was negative.

From a consideration of these and other studies, one gains the impression that, while the surface of normal tonsils occasionally harbors beta hemolytic streptococcus, the crypts of tonsils, especially if hyperplastic, are frequently reservoirs of this bacterium. Swabs from the nasopharynx, on the other hand, yield beta hemolytic streptococci only under disease conditions of the throat, as in acute tonsillitis or nasopharyngitis or as transients resulting from the overflow of a carrier state in the tonsil.

As the bacteriologic findings in this series of middle ear infections indicate that the beta hemolytic streptococcus is the probable causative organism in the ear discharge of over 50% of the cases, its presence in the throat normally and during disease has a direct bearing on this investigation.

The relation of the throat flora to the predominating organism in the ear discharge in acute cases at the time of the primary culture is given in table 3.

¹⁰ Jour. Am. Med. Assn., 1918, 71, p. 1813.

¹¹ Jour. Am. Med. Assn., 1918, 70, p. 1827.

¹² Jour. Infect. Dis., 1920, 27, p. 618.

¹³ Jour. Infect. Dis., 1919, 24, p. 386.

¹⁴ Jour. Am. Med. Assn., 1919, 73, p. 1050.

¹⁵ Bull. Johns Hopkins Hosp., 1921, 32, p. 33.

¹⁶ Arch. Int. Med., 1923, 32, p. 386.

Since these tabulations are on the basis of the nasopharyngeal or tonsil surface flora and not from the crypts, the results are more indicative of a deviation from the normal flora. It will be seen that there was a marked correlation between the throat and ear bacteria when the beta hemolytic streptococcus, pneumococcus or green streptococcus were the etiologic factors in the ear infection. Since the throats in these cases were either acutely inflamed at the time of the throat culture or had recently recovered from an infection, it seems logical to conclude that the beta hemolytic streptococcus and pneumococcus were of some etiologic significance in the throat inflammation. If serologic tests should prove that the organisms found in the ear and throat were identical, the source of the ear infection in these cases would be partially though not absolutely established. Whether the

TABLE 3

CORRELATION OF THE BACTERIA IN THE THROAT WITH THE PREDOMINATING ORGANISM IN THE EAR DISCHARGE

Bacteria	Incidence in		%
	Ear*	Throat*	
B. hem. strep.	44 cases	39 cases	90
Pneumococcus type 4.....	7 cases	7 cases	100
Green strep. (alpha).....	3 cases	3 cases	100
Staphylococcus	20 cases	4 cases	20
Diphtheroid bacillus	3 cases	0 cases	
Staphylococcus and diphtheroid bacillus.....	3 cases	1 case	25

* Same case.

mode of transmission to the middle ear is by way of the lymphatics or air borne through the lumen of the Eustachian tube, remains to be investigated. The green (alpha) streptococcus is normally present in the throats of healthy persons unless overgrown temporarily by a more virulent organism.

In cases in which staphylococci or diphtheroid bacilli were the apparent etiologic factors in the ear discharge, the same varieties were found in the throat in very few instances. The throat flora in these cases consisted mainly of green streptococcus, mixed occasionally with gram-negative cocci such as are usually found as normal inhabitants of the throat. It is noteworthy that in only two of these cases could cultures of the beta hemolytic streptococcus be obtained in small numbers from the throats. In only a few cases yielding staphylococcus or diphtheroid bacilli were cultures made more than once, due to the fact that they were extremely mild and the patients did not return to the

clinic. No interferences can be made in regard to the source of infection in these cases since throat cultures were not taken prior to the ear conditions and since these bacteria are known to occur on the surface of the skin.

STUDY OF THE EAR AND THROAT FLORA MADE DURING THE
SUBSEQUENT COURSE OF THE DISEASE

Cultures were made in a considerable number of cases at intervals over a period of time in order to study the change in the type of organisms in the ear and also in the throat as the clinical conditions improved or became chronic.

TABLE 4
BACTERIOLOGIC COURSE IN CASE 13

Case 13, aged 4 years, cold one month previous.

Date	Left Ear	Throat	Clinical Notes
Jan. 28	B. hem. strep. 84% Staph..... 20%	Green strep... 80% B. hem. strep. 20%	Spontaneous rupture; profuse discharge on admission
Feb. 9	Staph..... 50% B. hem. strep. 20% Green strep... 20% Diph. bac..... 10%	Green strep... 70% Staph..... 20% B. hem. strep. 10%	Feb. 4, in hospital as pneumonia suspect; temperature high, other ear suspicious; Feb. 9, no pneumonia foci, temperature and right ear normal
Feb. 25	Diph. bac..... 90% Staph..... 10%	Green strep... 90% Staph..... 10%	Large amount of pus in ear
March 6	Tonsillectomy and adenoidectomy		
March 21	Diph. bac..... 90% Staph..... 10%	Gamma strep. 70% Green strep... 20% Diph. bac..... 10%	Small amount of mucoid discharge from ear
April 25	Few large gram-positive cocci	Green strep... 100%	Small amount of discharge; hearing normal

Tables of cases 13, 17, 19, 21 and 51 are chosen as typical of the bacteriologic course of acute middle ear infections caused by the beta hemolytic streptococcus.

Cases 19 and 21 cleared up rapidly with no complications, cultures showing that the beta hemolytic streptococcus in the ear and in the throat was supplanted after a short time by types of organisms which are found normally, although case 21 was carrying a small proportion of beta hemolytic streptococcus in the ear and throat when cultures were last made. Case 13 was more refractory. After one month had elapsed, the beta hemolytic streptococcus had disappeared from the ear discharge and from the throat as far as cultures could determine, the ear at the time yielding diphtheriod bacilli and staphylococci. Case 17

was observed for a short time, but is interesting in that it shows an infection of the right ear yielding a diphtheroid bacillus 3 days after the beta hemolytic streptococcus was found in the left ear. Case 51 is a postoperative otitis media in which the right ear became involved acutely 2 days after the left.

TABLE 5
BACTERIOLOGIC COURSE IN CASE 17

Case 17, aged 12 years, pain in left ear following cold, drum ruptured 48 hours later; tonsils out.

Date	Right Ear	Left Ear	Nose	Throat	Clinical Notes
Feb. 1	B. hem. strep. 80% Staph. 20%	B. hem. strep. 100%	B. hem. strep. 80% Green strep. 20%	Paracentesis of left ear 3 weeks after spontaneous rupture
Feb. 4	Diph. bac. 100%	Staph. 60% B. hem. strep. 40%	B. hem. strep. 100%	Paracentesis of bulging right ear; small spontaneous rupture 24 hours before
Feb. 6	Diph. bac. 100%	B. hem. strep. 100%	Left drum normal; slight bulging of right

TABLE 6
BACTERIOLOGIC COURSE IN CASE 19

Case 19, aged 1 year; Jan. 26, had fever and pharyngitis; Jan. 31, spontaneous rupture of left drum; Feb. 1, spontaneous rupture of right drum.

Date	Right Ear	Left Ear	Throat	Clinical Notes
Feb. 3	B. hem. strep. 90% Staph..... 10%	B. hem. strep. 100%	B. hem. strep. 60% Green strep... 20% Staph..... 20%	Tonsils large and reddened, has acute nasopharyngitis
Feb. 9	B. hem. strep. 60% Staph..... 40%	B. hem. strep. 100%	Green strep... 50% Staph..... 40% B. hem. strep. 10%	Ears draining O.K., temperature normal
Feb. 20	Staph..... 80% Diph. bac..... 20%	Green strep... 70% Staph..... 20%	
April 4	Green strep... 80% Staph..... 20%	Drums O.K.

SECONDARY ACUTE INFECTIONS CAUSED BY A DIFFERENT BACTERIUM

In one of the cases, case 40, there was a recurrence of symptoms in the other ear 3 months later, in which a different bacterium was found.

Case 34 is of interest as it shows a second infection resulting in a fatal meningitis after recovery from a mild ear infection one month previous.

A review of the bacteriologic findings in the cases cultured over a period of from 1 week to 10 months shows that, in the majority of cases, cultures from the ear and throat taken from 3 to 6 days after paracentesis or rupture exhibited a marked change in the relative proportions of the different bacterial varieties. For example, the beta hemolytic streptococcus which made up a high percentage of the total

TABLE 7
BACTERIOLOGIC COURSE IN CASE 21

Case 21, aged 12 years; tonsils out; Jan. 28, severe cold; Feb. 1, blowing nose "forced something into ear," 48 hours later the drum ruptured.

Date	Right Ear	Nose	Throat	Clinical Notes
Feb. 4	B. hem. strep. 80% Staph..... 20%	B. hem. strep. 100%	Green strep... 60% B. hem. strep. 30% Staph..... 10%	Drum ruptured 24 hours previous
Feb. 6	Staph..... 70% B. hem. strep. 30%	B. hem. strep. 80% Green strep... 20%	Ear draining O.K.
Feb. 11	Staph..... 50% B. hem. strep. 30% Green strep... 20%	B. hem. strep. 50% Green strep... 50%	Discharge much less
Feb. 25	Staph..... 95% B. hem. strep. 5%	Green strep... 80% B. hem. strep. 20%	Drum normal

TABLE 8
BACTERIOLOGIC COURSE IN CASE 51

Case 51, aged 4½ years; postoperative otitis media; tonsillectomy and adenoidectomy March 22; edema of uvula.

Date	Right Ear	Left Ear	Throat	Clinical Notes
March 25	B. hem. strep. 100%	B. hem. strep. 60% Green strep... 30% Gamma strep. 10%	Left drum grey and bulging, paracentesis, much fluid pus
March 27	B. hem. strep. 100%	B. hem. strep. 70% Green strep... 30%	Right drum grey and bulging, paracentesis
April 29	Staph..... 60% B. hem. strep. 30%	Staph..... 60% B. hem. strep. 30% Diph. bac..... 10%	B. hem. strep. 20% Green strep... 80%	Throat healed, small amount of discharge from both ears

flora of the ear discharge at first began to disappear, and other organisms, such as staphylococci and diphtheroid bacilli, supplanted it. The clinical condition likewise improved. In secondary cultures from the throats, the normal inhabitants, such as green (alpha) streptococci and gram-negative cocci, came to the foreground as the proportion of beta hemolytic streptococci became lower. A few cases were encountered, however, as for example cases 13, 12 and 29, in which the hemolytic streptococcus persisted for a longer time, and recovery was delayed.

TABLE 9

BACTERIOLOGIC COURSE IN CASE 40

Case 40, aged 31 years. August, 1920, left mastoid operated; February, 1921, pain in right ear and discharge.

Date	Right Ear	Left Ear	Throat	Clinical Notes
March 4, 1922	Staph..... 100%	Green strep... 60% Gamma strep. 30% Gram pos. bac..... 10%	Tonsils and adenoids out, much lymphoid hypertrophy on posterior pharyngeal wall
April 1	* B. hem. strep. 100%	B. hem strep. 70% Green strep... 30%	March 30, acute nose and throat, otitis media, spontaneous rupture; April 1, bulging mastoid wound operated on
April 28	Staph..... 98% B. hem. strep. 2%	Green strep... 100%	
June 12	Pneumococcus Group 4..... 100%	Staph..... 100%	Pneumococcus Group 4..... 100%	Acute throat; right ear discharging

* Culture from mastoid.

TABLE 10

BACTERIOLOGIC COURSE IN CASE 34

Case 34, aged 6 months. Postmortem findings: Heart blood and material from mastoid contaminated. Pneumococcus type 1 isolated from a localized meningitis about 5 cm. above the left internal auditory meatus lying over the prerolandic area. The internal meatus and tegmen tympani were microscopically involved, and there was no connection between the leptomeningitis and the ear or mastoid.

Date	Right Ear	Left Ear	Throat	Clinical Notes
Feb. 24	No growth	No growth	Staph..... 60% Green strep... 40%*	Acute upper respiratory infection paracentesis of both ears
March 8	Staph..... 100%	Staph..... 60% Pneumococcus type 1..... 30% Green strep... 10%	Ears discharged until March 6, when patient was sent home recovered
March 22	Pneumococcus type 1..... 40% Staph..... 60%	Pneumococcus type 1..... 40% Green strep... 40% Staph..... 20%	Patient readmitted to hospital with temp. 39 C.; paracentesis of bulging left drum
March 23	Pneumococcus type 1..... 95% Staph..... 5%	Staph..... 80% Pneumococcus type 1..... 20%	Pneumococcus type 1..... 100%	Right ear opened because of progressing otitis media
March 24	Patient died with signs of meningitis without any signs of mastoid involvement.			

* Since a mouse inoculation was not made, the presence of some pneumococcus type 1 organisms cannot be ruled out.

Since the secondary organisms in the ear discharges in these cases were the same varieties as were found in the milder cases, the longer duration cannot be attributed to the presence of any specific secondary organism, unless certain strains of these bacterial varieties should prove to be more invasive than others.

BILATERAL INFECTIONS

There were 23 cases of acute involvement in both ears, including one case in which the infections of the right and left ears occurred 3 months apart (table 11).

Case 40, in which the second ear was involved 3 months after the first, is not included in this table.

TABLE 11
BACTERIAL VARIETIES IN 22 CASES OF BILATERAL OTITIS MEDIA

Right Ear	Left Ear	No. of Cases
B. hem. strep.	B. hem. strep.	9
Diphtheroid bacillus.....	B. hem. strep.	1
B. hem. strep.; staphylococcus.....	Staphylococcus.....	1
Pneumococcus group 4.....	Pneumococcus group 4.....	1
Staphylococcus.....	Staphylococcus.....	2
Staphylococcus.....	Diphtheroid bacillus.....	1
Staphylococcus.....	Staphylococcus; green (alpha) strep.	2
Staphylococcus.....	Diphtheroid bacillus; staphylococcus.....	1
Green (alpha) strep.	Green (alpha) strep.; staphylococcus.....	1
Green (alpha) strep.	Green (alpha) strep.	2
Sterile.....	Sterile.....	1

A careful scrutiny of the histories available shows that in the majority of bilateral cases the height of infection of the two ears was not simultaneous, but that one ear reached the acute stage several days before the other. It is interesting to consider, although difficult to prove, whether both ears were infected simultaneously, the second one with a lighter infection; whether the second was infected from the throat at a later date, or transferred from the infection of the first ear either by way of the Eustachian tube and throat or the lymphatics.

MASTOID INFECTIONS

There were 5 cases in which mastoid operations were performed. The organisms recovered from the mastoids were as follows: Beta hemolytic streptococcus from 3 cases, pneumococcus type 3 from 1 case; and a slowly growing green streptococcus from both ears in a bilateral mastoid infection.

SUMMARY OF RESULTS WITH REFERENCE TO DIFFERENT
BACTERIAL VARIETIES

In summarizing the results, we find that the beta hemolytic streptococcus was found in 53% of the acute cases and was therefore the organism of greatest significance in a study of acute otitis media. This bacterium was chosen for concentrated investigative work which will be discussed later.

Green (alpha) streptococci were found in pure culture in 5 cases. In the cases of the bilateral mastoid infection, the streptococcus was an extremely small, slow growing organism which produced a small amount of green discoloration on the blood plate. It died off quickly on artificial cultivation. Since the green streptococcus is a normal inhabitant of the throat, its presence in the ear under certain conditions is not surprising.

Pneumococci were the etiologic factors in a relatively smaller number of these cases. It is probable that during a period of acute respiratory disease due to this organism, a higher percentage of pneumococcic otitis media would be encountered. The secondary type 1 infection has been described above. The case due to the pneumococcus type 3 or *Pneumococcus mucosus* infection was severe. The acute middle ear infection was followed one month later by an infection of the mastoid. Mastoidectomy was performed and showed that the entire mastoid from the tip well into the zygoma was filled with pale jelly-like granulations with total destruction of the cell walls. The drum had been denuded over an area about 0.75 cm. in diameter and the sinus uncovered for 1.5 cm. The wound healed in 61 days. The discharge from the middle ear did not stop for 40 days after operation, and one paracentesis was necessary. The hearing was 2 feet for spoken voice with signs of nerve deafness, but as the patient had not been treated previous to the acute attack, no conclusions can be drawn. The remaining pneumococcus cases were due to group 4 and ran uncomplicated courses.

Staphylococci occurred in pure culture in the primary ear discharge from 22% of the acute cases, and mixed with other organisms in small or large proportions in about 70%.

The ratio of the different chromogenic varieties were: white, 30%; pale yellow, 20%; deep yellow, 50%. Whether these organisms were the true causative agents in the cases in which they were found in pure culture, or whether they were canal contaminants which had overgrown and obscured the true etiologic organisms, is debatable. The

mildness of symptoms in these cases would suggest that a bacterium of low virulence was the factor.

Diphtheroid bacilli, although frequent secondary invaders in the acute cases, which were due primarily to other organisms, were found in the original discharge in only 8 cases, in 5 of which they were mixed with other organisms. The morphologic and cultural characteristics and the possible significance of this group of organisms will be discussed later in connection with the subject of chronicity.

It is noteworthy that no Friedländer bacilli were encountered in this study.

CULTURAL AND SEROLOGIC DIFFERENTIATION OF THE BETA HEMOLYTIC STREPTOCOCCI OF ACUTE MIDDLE EAR INFECTIONS

Since the beta hemolytic streptococcus was found to be the probable etiologic agent in 53% of the acute middle ear infections, in 90% of which it was present in the throats as well, this organism was used for detailed study in an attempt to throw light on the following points:

1. Did a pure culture cause the ear infection? If so, all the strains isolated from a single ear discharge will be similar.
2. What is the relative prevalence of the different fermentative groups?
3. Are both ears in cases of double otitis media infected by the same variety?
4. Are the hemolytic streptococci found in the ear and throat in individual cases at the time of the primary culture similar?
5. Did the same variety of streptococcus persist in the ear and in the throat during the period of observation?
6. If a change in type in the ear discharge occurred, did this change have any significance when correlated with the clinical history, i. e., in regard to the severity, duration of symptoms and chronicity?
7. Could any definite group or groups be demonstrated among the strains from the different cases which might indicate that certain varieties of beta hemolytic streptococcus were the causative factors in acute middle ear infections?
8. Are the strains of beta hemolytic streptococcus isolated from the large number of acute middle ear infections which occurred during the epidemic of respiratory disease in the winter of 1922 similar or identical? If serologic tests should prove them to be alike, it might

suggest that one is dealing with an epidemic strain or with a common virulent secondary invader.

Present knowledge indicates that the beta hemolytic streptococcus is not a single bacterial type, but the various strains classed under this general head can be differentiated by cultural and serologic tests. The answers to the points tabulated above could only be rendered through detailed bacteriologic studies.

FERMENTATION REACTIONS

The ability to ferment certain carbohydrates was the first reaction employed in differentiating these strains of beta hemolytic streptococci. Although the final proof of similarity or dissimilarity of streptococci cannot be made on a basis of the fermentation results, this test is of value as a preliminary method of classification.

The carbohydrates used were lactose, salicin, mannite, raffinose, inulin and saccharose, following the Holman¹⁷ classification as revised by Brown.⁸ The medium consisted of sugar free veal infusion to which 1.5% peptone, 0.5% agar, 1% Andrade's indicator and 1% of the carbohydrate was added. Confirmatory tests were carried out in Hiss serum water with 1% of the carbohydrate and Andrade's indicator. Observations were made after 2, 7, and 14 days' incubation. Four strains were isolated from each specimen except those showing a difference in colony characteristics in blood-agar plates. In such instances, 3 of each variety were isolated and tested.

The result of the fermentation tests showed that all strains (tentative answer to question 1) isolated from each primary ear discharge were similar in their fermentative reactions, or, in other words, there was apparently one variety causing the infection.

The proportion of the different fermentative groups (question 2) found in the primary specimens of 53 acute ear discharges was as follows: pyogenes group, 45, or 85%; infrequens group, 5, or 9.4%; equi group, 3, or 5.6%.

The fermentation reactions on the strains from 9 cases which yielded beta hemolytic streptococcus in both ears (question 3) showed that in 8 cases, strains of the same fermentative group occurred in both ears, whereas in one case, all strains isolated from the right ear belonged to the pyogenes group and all strains isolated from the left ear belonged to the infrequens group. This case will be discussed in detail.

¹⁷ Jour. Med. Res., 1916, 34, p. 377.

The fermentation reactions were of value as a preliminary method in determining the relationship of the beta hemolytic streptococcus found in the throat with that occurring in the ear (question 4). Table 12 shows the correlation of strains of beta hemolytic streptococcus from the ear and throat on a basis of their carbohydrate fermentation.

PERSISTENCE OF FERMENTATIVE GROUPS OF BETA HEMOLYTIC
STREPTOCOCCUS IN THE EAR AND THROAT OVER
A PERIOD OF TIME

Twenty-three of the cases in which the beta hemolytic streptococcus was found in the ear discharge were cultivated over a period of from 1 week to 10 months. With the exception of 2 cases, nos. 12 and 29, strains belonging to the same fermentative groups were isolated from the ear discharge and from the throat, as long as the streptococcus persisted (question 5).

TABLE 12
FERMENTATIVE GROUPS OF BETA HEMOLYTIC STREPTOCOCCUS IN THE EAR AND THROAT AT
THE TIME OF THE PRIMARY CULTURE

One Ear Involved	Both Ears Involved	Throat Culture	No. of Cases
Strep. pyogenes.....	Strep. pyogenes.....	Strep. pyogenes.....	27
	Strep. pyogenes.....	Strep. pyogenes.....	6
	Strep. pyogenes, right ear; Strep. infrequens, left ear	Strep. infrequens.....	1
Strep. pyogenes.....		No B. hem. strep.	5
Strep. infrequens.....		Strep. infrequens.....	2
	Strep. infrequens.....	Strep. infrequens.....	1
	Strep. equi.....	Strep. equi.....	1
Strep. equi.....		No B. hem. strep. at first, Strep. pyogenes at later culture	1

In 17 of these cases, the hemolytic streptococci isolated over the entire period belonged to the pyogenes group, the type most frequently found in pyogenic conditions, and possibly capable of further differentiation by serologic tests. Of the other 4 cases, 3 yielded mannite fermenting (infrequens group) strains, while all the strains from the 4th case, 20, isolated from the ear and throat were nonlactose fermenters (equi group). The continued presence in these four cases of hemolytic streptococci possessing relatively unusual fermentative characteristics is strong presumptive evidence of the similarity of the strains in each of these cases.

The 2 cases, 12 and 29, which contained hemolytic streptococci belonging to different fermentative groups, are worthy of detailed study. Although the dissimilar fermentation results would suggest a lack of

relationship between the strains, serologic tests were necessary in order to prove that the apparent difference was not the loss or acquisition of certain fermentative characteristics by the same strain. Individual charts of cases 12 and 29 may aid in interpreting the bacteriologic results in the light of the clinical histories (question 6).

Discussion of Case 12.—It is difficult to attempt an interpretation of this unusual case, since, unfortunately, both ears had been draining for

TABLE 13
BACTERIOLOGIC COURSE IN CASE 12

Case 12, aged 3 years; patient in hospital with bronchitis two months previous; has rickets.

Date	Right Ear	Left Ear	Throat	Clinical Notes
Jan. 28	B. hem. strep. (pyogenes).. 100%	B. hem. strep. (infrequens) 40% Green strep... 20% Staph..... 40%	B. hem. strep. (infrequens) 60% Green strep... 20% Gram neg. cocci..... 20%	Spontaneous rupture of both drums one month ago; large amount of purulent discharge from both ears
Feb. 8	B. hem. strep. (infrequens) 80% (equi)..... 10% Diph. bac..... 10%	B. hem. strep. (infrequens) 80% Staph..... 20%	B. hem. strep. (infrequens) 60% Green strep... 20% Gram neg. coc. 10% Staph..... 10%	Both ears discharging, no temperature
Feb. 15	Diph. bac..... 99% B. hem. strep. (equi)..... 1%	B. hem. strep. (infrequens) 50% Diph. bac..... 50%	B. hem. strep. (infrequens) 80% Green strep... 10% Staph..... 10%	Both ears discharging, no temperature
Feb. 25	Diph. bac..... 100%	Diph. bac..... 80% Staph..... 20% Also fusiform bac. in direct smear	Green strep... 70% Diph. bac..... 30%	Both ears discharging, no temperature
March 6	Tonsillectomy and adenoidectomy			
March 21	Diph. bac..... 99% B. hem. strep. (equi)..... 1%	Diph. bac..... 90% B. hem. strep. (equi)..... 10%	B. hem. strep. (pyogenes).. 70% Green strep... 10% Gamma strep. 10% Diph. bac..... 10%	
April 28	Diph. bac..... 90% Staph..... 10%	Green strep... 90% Staph..... 10%	Right ear normal, notes noises O.K.; left ear, stringy muco-pus; no odor; lymphoid hyperplasia in throat less

several weeks at the time the first cultures were taken. We might assume that Streptococcus pyogenes, which was found in pure culture in the right ear, had been the etiologic factor in the left ear, as well as in the bronchitis of a month previous. In some way, the throat had been reinfected by a different variety of beta hemolytic streptococcus (of the infrequens group), which being a virulent strain also, had supplanted the hypothetical pyogenes strain in the left ear. By the time

of the second culture, the right ear as well as the left yielded the infrequens strain, which still persisted in the throat. Due to the fact that the infrequens strain had overgrown the original streptococcus to which the body possibly had become immune, this variety (infrequens) persisted for a week longer, being supplanted at that time by diphtheroid bacilli in both ears.

The presence of the few colonies of beta hemolytic streptococcus of the equi group can be accounted for only as a chance contaminant. Fifteen days after tonsillectomy the throat contained streptococci of the pyogenes group which proved by agglutination tests to be different from the pyogenes strain found in the original discharge of the right ear.

The fact that a second virulent variety of beta hemolytic streptococcus had gained a foothold in this case might account for the prolonged duration of symptoms lasting at least 3 months. This patient had left the city, so the ultimate outcome is not available at present. The clinical and bacteriologic findings of the case when last seen would indicate a prognosis of chronic discharge in the left ear.

The bacteriologic findings in case 29 showed a change in the variety of hemolytic streptococcus in the ear discharge after 7 days, and at a later date showed a mixture of hemolytic streptococci belonging to 2 different fermentative groups in the ear. The objective results in this case are given without attempting to interpret them. The clinical notes would seem to offer certain possible explanations.

The fact that, in this case as in case 12, the ear had ruptured spontaneously and had been discharging for a considerable time before medical attention had been given, is suggestive that delay in caring for ear infections may be serious, for reinfection and a chronic process may result.

SEROLOGIC RELATIONSHIP OF STRAINS OF BETA HEMOLYTIC STREPTOCOCCUS ISOLATED FROM THE EAR DISCHARGE AND FROM THE THROAT IN THE INDIVIDUAL CASE

The differentiation of the beta hemolytic streptococci up to this point had been on the basis of their fermentative characteristics. As stated previously, these results are inadequate as a final test of antigenic similarity. Dochez, Avery and Lancefield¹⁸ were the first to show that strains of hemolytic streptococcus, culturally similar, could be differentiated by agglutination and protection methods. They were able to

¹⁸ Jour. Exper. Med., 1919, 30, p. 179.

distinguish 5 definite groups besides a heterologous group among a large number of pyogenes strains, obtained mostly during the epidemic at one of the war camps. Since strains of streptococcus biologically similar can thus be differentiated, immunologic experiments were carried out to prove the antigenic identity of the strains of streptococcus in an individual case of otitis media.

The method of approach was as follows: Streptococcus pyogenes strains from the original ear discharge from each case were tested with

TABLE 14
BACTERIOLOGIC COURSE IN CASE 29

Date	Right Ear	Throat	Clinical Notes
Feb. 13	B. hem. strep. (equi)..... 80% Gram-neg. bacilli..... 20%	Ear had been discharging for 14 days
Feb. 21	B. hem. strep. (infrequens) 60% Staph..... 30% Green strep... 10%	Green strep... 80% Gram-pos. cocci..... 20%	Feb. 18, no discharge from ear, but marked swelling over mastoid; Feb. 21, ear began discharging again and relieved pain
Feb. 24	B. hem. strep. (infrequens) 40% (pyogenes)..... 40% Staph..... 40% Large gram-pos. bac..... 20%	Green strep... 60% Gram-neg. cocci..... 40%	Profuse grey discharge; drum red and bulging; reincised
Feb. 28	B. hem. strep. (infrequens) 40% (pyogenes)..... 40% Large gram-pos. bac..... 40% Staph..... 20%	B. hem. strep. (pyogenes).. 40% Pneum. gr. 4.. 20% Green strep... 10% Staph..... 30%	Ear still discharging; free from fever
March 14	B. hem. strep. (pyogenes).. 30% Large gram-pos. bac..... 60% Staph..... 10%	Green strep... 50% Pneum. gr. 4.. 50%	Reincised bulging drum
April 4	Staph..... 50% Large gram-pos. bac..... 50%	Green strep... 60% Gram-pos. bacilli..... 40%	Thick mucoid discharge from ear

serums immune to the Dochez, Avery and Lancefield¹⁸ pyogenes strains 3, 23, 32, 84 and 273. Only one case, 52, showed any marked degree of agglutination, and further tests proved that all of the strains isolated from this case were identical with the Dochez, Avery and Lancefield¹⁸ type strain 32. Immune rabbit serums were prepared for one pyogenes strain from the original ear discharge of cases 2, 12, 19 and 29 and later for cases 61, 65, 69 and 90. An antiserum was prepared also for the infrequens strain from the left ear in case 12. Strains which tended to grow diffusely in beef broth and preferably from cases in which both ears were involved were chosen for serum production.

METHODS OF AGGLUTINATION AND ABSORPTION TESTS

The tendency of some strains of streptococci to clump spontaneously when grown in broth is the cause of considerable difficulty in carrying out agglutination tests. In this investigation, several different procedures were used, depending on the degree of spontaneous agglutination of the various strains.

A number of the strains produced a diffuse cloud when grown for 18 hours in beef broth (containing 1% peptone, 0.5% NaCl P_H 7.8). This broth culture diluted with sterile beef broth to the desired density was used for agglutination. Other strains which had a slight tendency to clump when grown in the same medium were satisfactory when shaken thoroughly by hand, allowed to stand for 2 hours, and the upper portion decanted off for use. When the preceding methods were not applicable, a modification of the method described by Norton¹⁹ was used. It consisted in growing the cultures in beef broth to which 1% dextrose (instead of dextrin) and a few drops of ascitic fluid water (1 part ascitic fluid and 3 parts distilled water) had been added just before inoculation. After 18 hours' growth, the cultures were centrifugalized and beef broth added slowly to the sediment, until cloudy enough for use. The strains isolated from a small minority of the cases were so flocculent that suitable agglutinating antigens could not be obtained by any of the foregoing methods.

The serum to be used in the test was diluted with beef broth and tubes set up containing 0.1 c.c. of serum dilution and 0.9 c.c. of the agglutinating antigen. These tubes were placed in the water bath at 55 C. and readings made at the end of 2 hours.

ABSORPTION TESTS

A broth culture of the strain to be used for absorption was packed by centrifugalization in a graduated centrifuge tube. A dilution of 1:15 of the serum to be absorbed was added to the packed bacteria in the proportion usually of 1 part bacteria to 10 parts of serum dilution. Absorption was carried on for 3 hours in a water bath held at 45 C., the tubes being shaken at 15 minute intervals. The mixture was placed in the icebox overnight. In the morning, it was centrifugalized, and agglutination tests were made with the supernatant fluid as well as with the unabsorbed serum as a control.

The result of the direct agglutination and agglutinin absorption tests of *Streptococcus pyogenes* cultures from case 2 proved that all of the strains isolated from the ear and from the throat over a period of 6 weeks were alike. The pyogenes strains from the right and left ears of case 19 were identical, as well as those in the throat. The protocols of the agglutinin absorption tests are not included. Since the ear infections in these cases were immediately subsequent to an acute infection of acute tonsillitis and cervical adenitis in case 2 and acute nasopharyngitis in case 19, the isolation of identical strains from the ear discharge and from the throat indicates the source of the ear infections in these cases.

Cases 12 and 29 are more involved, since both cases contained 3 different fermentative groups of beta hemolytic streptococcus. Table 16 shows the result of the direct agglutination of all the strains isolated

¹⁹ Jour. Am. Med. Assn., 1921, 76, p. 1753.

from case 12 with antiserums prepared for 12A, the pyogenes strain from the right ear, and for 12B, the infrequens strain from the left ear. It will be noted that all of the pyogenes strains from the preliminary discharge in the right ear were agglutinated to the titer of the serum, but that the pyogenes strains found in the throat 2 months later were negative on direct agglutination. All of the strains of the infrequens group persisting in the ear and throat for a period of 3 weeks were similar by direct agglutination, while strains of the equi group showed no reaction with either the pyogenes or infrequens antiserums.

Agglutinin absorption tests were carried out as a final indication of identity or nonidentity. Table 17 shows the result of the agglutinin

TABLE 15
DIRECT AGGLUTINATION RESULTS OF STREPTOCOCCUS PYOGENES STRAINS FROM
CASES 2 AND 19

Case 2				Case 19			
Strains	Source	Date	Agglutination Immune Serum 2A4	Strains	Source	Date	Agglutination Immune Serum 19B
A 4, 5	Left ear	Jan. 23	4000	B 1, 2, 3, 5	Left ear	Feb. 3	2000
B 1, 2, 3	Throat	Jan. 23	4000	C 1, 2, 3	Right ear	Feb. 3	2000
C 1, 2, 3	Left ear	Jan. 27	4000	D 1, 2, 3	Throat	Feb. 3	2000
D 3, 4, 8	Left ear	Jan. 30	4000	E 1, 2, 3	Left ear	Feb. 9	2000
F 1, 2, 3	Throat	March 2	4000	F 1, 2, 3	Right ear	Feb. 9	2000
G 1, 2	Throat	March 6	16000	G 1, 2	Throat	Feb. 9	2000
G 3*	Throat	March 6	0 (100)	G 3*	Throat	Feb. 9	0 (100)

* Strain of green (alpha) streptococcus.

Figures give the highest dilution in which complete agglutination took place; in case of no reaction the lowest dilution tested is given in parenthesis.

The reactions of the strains from each specimen were markedly similar and therefore the readings given are the average of the strains tested.

absorption tests of certain representative pyogenes and infrequens strains from case 12.

We find, therefore, that the infrequens strains are all identical, that the pyogenes strain found in the original discharge in the right ear is different antigenically as well as fermentatively from the infrequens strain in the left ear, and that the pyogenes strain O present in the throat is different from pyogenes strain A found in the right ear two months previously.

By direct agglutination and agglutinin absorption, the strains of case 29 were true to their fermentative classification; that is, all of the infrequens strains were similar and all of the pyogenes strains were similar, with no evidence of cross reaction between them. The infrequens strains of case 29 were identical with the infrequens strains of

case 12, so the antiserum prepared for 12B, was available for testing the infrequens strains of case 29.

Direct agglutination and agglutinin absorption tests were carried out on the strains from cases 61, 65, 69 and 90 with antisera prepared for one original ear culture of each case, but the protocols will not be included for lack of space. The results proved the identity of all the strains isolated from each individual case.

A summary of the agglutination results in regard to the antigenic similarity of all the strains of beta hemolytic streptococcus isolated from an individual case shows that in 7 cases, 3 of them bilateral, the strains of beta hemolytic streptococcus occurring in the ears and throat in each

TABLE 16
DIRECT AGGLUTINATION RESULTS OF BETA HEMOLYTIC STREPTOCOCCUS STRAINS FROM CASE 12

Strain	Source	Date	Fermenta- tive Group	Agglutination Results		
				Immune Serum 12A Pyogenes	Immune Serum 12B Infrequens	Control
A 1, 2, 5, 6	Right ear	Jan. 28	Pyogenes	10000	200	0
B 1, 2, 3	Left ear	Jan. 28	Infrequens	0 (200)	2000	0
C 1, 2, 3	Throat	Jan. 28	Infrequens	0 (200)	2000	0
D 1, 2	Right ear	Feb. 6	Infrequens	0 (200)	2000	0
D 3, 4	Right ear	Feb. 6	Equi	0 (200)	0 (200)	0
E 1, 2, 3	Left ear	Feb. 6	Infrequens	0 (200)	2000	0
F 1, 2, 3	Throat	Feb. 6	Infrequens	0 (200)	2000	0
G 1*	Right ear	Feb. 15	Equi	0 (200)	0 (200)	0
H 1, 2, 3	Left ear	Feb. 15	Infrequens	0 (200)	2000	0
J 1, 2, 3	Throat	Feb. 15	Infrequens	0 (200)	2000	0
N 1, 2, 3	Right ear	March 21	Equi	0 (200)	0 (200)	0
P 3, 4	Left ear	March 21	Equi	0 (200)	0 (200)	0
O 1, 2, 3	Throat	March 21	Pyogenes	0 (200)	0 (200)	0

* Only one hemolytic colony on plates.

Figures give the highest dilution in which complete agglutination took place; in case of no reaction the lowest dilution tested is given in parenthesis.

case over a considerable period of time were identical. In two cases, 12 and 29, there was a difference in the groups of beta hemolytic streptococcus in the ear and throat, and a change of the group in the ear discharges. The conclusion seems justified, therefore, that in the majority of acute ear infections the type of beta hemolytic streptococcus occurring in the original ear discharge will be found also in the throat (question 4), the ear infection having been subsequent to an infection of the nose or throat in most instances and that the same type will persist in the ear until supplanted by other organisms in cases which run an uncomplicated course of recovery in a moderately short time (question 5). The prolonged duration of the disease in certain cases, such as no. 12 and 29, may be due in part to the change in the variety of beta hemolytic streptococcus.

SEROLOGIC GROUPING OF STRAINS OF BETA HEMOLYTIC
STREPTOCOCCUS FOUND IN THE EAR DISCHARGE OF
THIS SERIES OF ACUTE EAR INFECTIONS

(Questions 7 and 8).

In the serologic work up to this point the relationship of hemolytic streptococci from individual cases was investigated. As stated previously, the broader aspect deserved consideration, whether any relationship existed among the beta hemolytic streptococci found in the ear discharge in different cases of otitis media. Serologic tests were carried out to determine whether there was any relationship among the ear strains as a whole and also whether the strains from cases which had occurred during the acute respiratory infections in the winter of 1922

TABLE 17
AGGLUTININ ABSORPTION RESULTS OF SELECTED BETA HEMOLYTIC STREPTOCOCCUS STRAINS
FROM CASE 12

Strains Used for Absorption		Immune Serum 12A Pyogenes		Immune Serum 12B Infrequens	
		Titer After Absorption		Titer After Absorption	
Strain No.	Fermentative Group	Absorbing Strain	Serum Strain	Absorbing Strain	Serum Strain
A1.....	Pyogenes	0 (150)*	0 (150)	2000
B1.....	Infrequens	0 (150)	10000	0 (150)
C1.....	Infrequens	0 (150)	0 (150)
D1.....	Infrequens	0 (150)	0 (150)
D3.....	Equi	0 (150)	10000	0 (150)	2000
G1.....	Equi	0 (150)	10000	0 (150)	2000
J1.....	Infrequens	0 (150)	0 (150)
O1.....	Pyogenes	0 (150)	10000

* Figures give the highest dilution in which complete agglutination took place; in the case of no reaction the lowest dilution tested is given in parenthesis.

were similar. Should the latter prove to be true, it might indicate the existence of an epidemic strain or a common secondary invader, which had a tendency to localize in the ear.

The result of the direct agglutination of the beta hemolytic streptococcus of the pyogenes group from 29 cases of acute ear infections with serums immune to 9 different ear strains is presented in condensed form in table 18. The strains from 11 additional cases could not be tested by direct agglutination due to extreme hyperagglutinability.

The results showed a marked degree of similarity in the direct agglutination reactions of strains from cases 2, 12, 63, 74, 92 and 99. Strains from case 41 were agglutinated to titer by serum immune to the pyogenes strain of case 29. Agglutinin absorption tests were carried

out as a final proof of identity. Since only one definite group had been encountered as a result of the direct agglutination tests, the antiserum for this group was the only one used to test the strains which were extremely unstable. Two strains which were negative on direct agglutination were included also. The results of the agglutinin absorption test is given in table 19

The result of this test showed that the strains which were agglutinated to the titer by direct agglutination were capable of absorbing the specific agglutinins from this serum, thus proving their identity. None

TABLE 18
CONDENSED TABLE OF THE DIRECT AGGLUTINATION RESULTS OF THE STREPTOCOCCUS
PYOGENES STRAINS ISOLATED FROM CASES OF OTITIS MEDIA

Case No.	Immune Serums								D. A. L. 32
	2	12	19	29	61	65	69	90	
2.....	4000*	10000	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)
8.....	0 (100)	0 (100)	0 (100)	0 (100)	100	0 (100)	0 (100)	100	0 (100)
12.....	4000	10000	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)
19.....	0 (100)	0 (100)	2000	0 (100)	0 (100)	0 (100)	100	0 (100)	0 (100)
29.....	0 (100)	0 (100)	0 (100)	2000	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)
41.....	0 (100)	0 (100)	0 (100)	2000	0 (100)	0 (100)	0 (100)	0 (100)	200
52.....	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)	4000
61.....	100	100	0 (100)	100	1000	0 (100)	0 (100)	100	200
62.....	4000	10000	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)
63.....	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)	2000	0 (100)	0 (100)	0 (100)
68.....	0 (100)	0 (100)	0 (100)	100	0 (100)	0 (100)	0 (100)	200	0 (100)
69.....	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)	800
74.....	4000	10000	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)
90.....	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)	2000	100
92.....	4000	10000	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)
99.....	4000	10000	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)
D. A. L. 32.....	0 (100)	4000

* Figures give the highest dilution in which complete agglutination took place; in case of no reaction the lowest dilution tested is given in parenthesis.

The results tabulated in this table show the average agglutination of 3 pyogenes strains isolated from the primary ear discharge of each case, except when spontaneous agglutination of one or more strains made them unsuitable for use.

Strains from cases 3, 13, 15, 17, 21, 24, 32, 51, 59, 67, 84, 89, and 98 gave negative reactions with all serums. Strains from cases 1, 35, 40, 45, 46, 48, 64, 66, 78, 81, and 83 were unsatisfactory for direct agglutination tests, due to extreme spontaneous agglutinability.

of the other strains exhibited any ability to absorb or reduce the specific agglutinins in the serum.

The strains of mannite fermenting hemolytic streptococci (infrequens) from 5 cases were tested by direct agglutination and agglutinin absorption with serum prepared for 12B, and Dochez, Avery and Lancefield¹⁵ type strain 60. The result showed that the infrequens strains of cases 12 and 29 were identical, but that strains from the other 3 cases gave negative reactions with both antisera.

All strains isolated from the 4 cases 63, 74, 92 and 99 the primary ear strains of which had proved to be identical with case 2, were tested

as additional proof of the type persistence in ear and throat in individual cases. Three of these cases were bilateral infections. The result of the tests proved that all strains isolated from these cases were similar, thus supporting the conclusion that in the majority of cases there is a persistence of the same variety of beta hemolytic streptococcus and that in bilateral infections the same streptococcus is usually the etiologic factor in both ears.

It is noted that among the 44 cases in which the beta hemolytic streptococcus was the apparent etiologic factor in the ear infection, 6 cases contained the identical serologic variety of *Streptococcus pyogenes*

TABLE 19

AGGLUTININ ABSORPTION RESULTS OF SELECTED STREPTOCOCCUS PYOGENES STRAINS WITH IMMUNE SERUM 2A4

Case and Strain No.*	Agglutination Titer		
	Unabsorbed Serum	Absorbed Strain	
		Absorbing Strain	Serum Strain
2 A4.....	4000	0 (150)
1 A1.....	Sp.	Sp.	4000
12 A1.....	4000	0 (150)	0 (150)
13 A1.....	0 (150)	0 (150)	4000
35 A1.....	Sp.	Sp.	4000
40 A1.....	Sp.	Sp.	4000
48 A1.....	Sp.	Sp.	4000
51 A2.....	Sp.	Sp.	4000
63 A1.....	4000	0 (150)	150
64 A3.....	Sp.	Sp.	4000
66 B2.....	Sp.	Sp.	4000
74 A1.....	4000	0 (150)	0 (150)
78 A2.....	Sp.	Sp.	4000
81 A1.....	Sp.	Sp.	4000
83 A2.....	Sp.	Sp.	4000
92 F1.....	4000	0 (150)	0 (150)
99 A1.....	4000	150	150

* Strains used for direct agglutination with unabsorbed serum and for absorption.

Figures give the highest dilution in which complete agglutination took place; in case of no reaction the lowest dilution test is given in parenthesis. Sp. indicates complete or partial spontaneous agglutinability.

in the ear discharge, that 2 other cases yielded pyogenes strains identical with each other, and that the infrequens strains of 2 cases were alike. The remaining cases showed no relationships in the tests carried out. It is not unlikely that other similarities might have been found among the remaining cases had more serums been prepared, but the practical application did not warrant further work, since so few identities had been found while using 9 immune serums for testing.

The question of contact among the cases whose strains proved to be similar was investigated, but no evidence to support such a possibility could be found. Moreover, the failure to find any marked degree of

relationship among the strains isolated during the period of acute respiratory disease in 1922 would indicate that no strain was encountered which had any epidemiologic significance (8th question).

THE SIGNIFICANCE OF SECONDARY ORGANISMS IN RELATION
TO CHRONICITY

Although the beta hemolytic streptococcus was the etiologic factor in the majority of the acute infections, and, therefore, the organism of greatest importance from a preventative and curative standpoint, many other aspects of the problem seemed worthy of investigation.

The results of the bacteriologic examination of the ear discharge from the cases studied over a period of time showed that the diphtheroid bacillus was a frequent secondary invader, but that it was found in the original discharge in only 8 instances, in 5 of which it was mixed with other organisms. In 56% of the cases observed consecutively, the diphtheroid bacillus was found associated with, or supplanting, the original organisms after periods of 1-3 weeks. It was cultivated from the discharge of 4 chronic cases.

The diphtheroid bacillus in relation to middle ear infection has been studied by many investigators, Graham-Smith,²⁰ Alice Hamilton,^{21, 23} Parker²² and others.

The morphology of the diphtheroid bacilli isolated from the different cases in this series was extremely varied; a single case occasionally showed 2 distinct morphologic types. Gram-positive bacilli, some exhibiting cross bar arrangement and varying from the short Hoffmann type to long regular bacilli with a number of metachromatic granules, were observed. In one instance, the organism from a chronic case was morphologically and culturally similar to true Klebs-Loeffler bacilli, but a virulence test on guinea-pigs proved it to be nontoxic.

The throat cultures of the cases harboring diphtheroid bacilli in the primary ear discharge failed, except in one instance, to show any of this group of organisms. Since diphtheroid bacilli are frequently found in small numbers in the throats of normal persons, it is probable that their presence in the throats in these cases had been obscured temporarily by the organisms causing the acute throat infections, especially as they were found in small proportions as the acute throat condition cleared up.

²⁰ Jour. Hyg., 1904, 4, p. 258.

²¹ Jour. Infect. Dis., 1907, 4, p. 326.

²² Jour. Med. Res., 1922, 43, p. 387.

The significance of diphtheroid bacilli as a group in relation to chronicity is worthy of further study, since it is often found in the discharge from chronic cases, and in this study has been shown to supplant the beta hemolytic streptococcus in the discharge from many acute cases. Whether this organism, primarily nonpathogenic, may be able through its products to exert a deleterious effect on the tissues of the middle ear which have become weakened by the acute inflammation, is a question. In a recent publication, Parker ²² has described an apparently new variety of diphtheroid bacillus isolated from otitis media, the majority following scarlet fever, which is toxic but not neutralizable by diphtheria antitoxin.

Staphylococci, both aureus and albus, were found in a number of chronic ear discharges and also mixed with other organisms in the later cultures from many acute cases. Because of the constant state of parasitism of this bacterium on the normal skin surfaces as well as on the mucous membranes of the nasal and buccal cavities, it is difficult to place any great significance on its presence in the chronic discharging ear. To what degree it may be the important factor in prolonging the ear condition of certain cases cannot be concluded from the data at hand.

Many other organisms, such as *B. pyocyaneus*, *B. proteus*, *B. coli*, etc., may be important factors in the ear discharges of a small proportion of chronic ear infections.

PRACTICAL VALUE OF THESE RESULTS WITH REFERENCE TO VACCINE THERAPY

It is evident from the results showing the heterogeneity of the bacterial varieties found in this series of cases that vaccine therapy in otitis media is a method of treatment not to be attempted blindly. In regard to streptococcus infections, the value of vaccines is a subject of debate at the present time. If beneficial results are obtained only when the specific organisms are used in treatment, the data presented in this report, namely, that the beta hemolytic streptococci found in the majority of ear discharges are immunologically different when tested by agglutination, would indicate that the use of a mixed stock vaccine would not be efficacious. If, however, further work should show that serologically different streptococci will give cross protection, then a mixed streptococcus vaccine might yield results. How far the "nonspecific" factor might influence the therapeutic results remains to be decided.

From the standpoint of specific therapy, the use of autogenous vaccines made from the streptococcus isolated from the original ear

discharge may be applicable, as it is shown that, in the majority of cases, the original streptococcus persists in the ear discharge and in the throat. However, the two cases, 12 and 29, in which the group of streptococcus changed, indicate that a thoroughly controlled course of vaccine treatment in any individual case entails serologic tests on the organisms isolated at succeeding weekly intervals.

The use of a diphtheroid vaccine in cases of suppurative otitis media, mostly postscarlatinal, has been reported by Hamilton.²³ The results as tested by opsonic determination suggested a certain benefit, and she concludes that "by the injections of dead cultures of the homologous strain, the patient's opsonin to that strain can be increased." She also studied the relationship of the pseudodiphtheria bacilli in otitis media, and claims to have differentiated two distinct groups when tested by bacteriolytic and agglutinative methods.

Since organisms of this group have been found in this study as the most frequent secondary invaders in the acute infections and as common inhabitants of the discharge from chronic cases, it would seem that further immunologic work leading to possible vaccine therapy might be of value. If certain acute cases could be immunized against this apparently pernicious secondary invader, results might be accomplished in preventing chronicity and deafness.

SUMMARY

The beta hemolytic streptococcus is probably the most important etiologic organism in the majority of acute middle ear infections in San Francisco. In a small percentage of cases, staphylococci, pneumococci, green (alpha) streptococci, and diphtheroid bacilli predominate.

The organisms obtained from 12 chronic cases, in pure or mixed culture, include diphtheroid bacilli, staphylococci, *B. pyocyaneus*, *B. proteus*, and *B. coli*.

A study of the throat flora in relation to the bacteria found in the ear discharges of acute middle ear infections reveals that in cases in which the beta hemolytic streptococcus, pneumococcus, or green (alpha) streptococcus are the apparent exciting factors in the ear discharge, the same bacterial species are found in the throat.

The indication as to the possible source of the ear infection, although not the final proof, has been secured by testing serologically the strains of beta hemolytic streptococcus of which cultures have been made simultaneously from the primary ear discharge and from the throat in the

²³ Jour. Infect. Dis., 1907, 4, p. 313.

individual case. The results obtained in the 9 cases studied with reference to this point show that the same serologic variety of streptococcus may be present in the throat and in the ear discharge.

In the majority of cases, cultures made from the ear discharge several days or a week after paracentesis show that the apparent etiologic bacterium has become reduced in number, while the proportion of other varieties has increased.

Two cases have been encountered in which the beta hemolytic streptococcus has persisted in the ear discharge over a prolonged period. Further tests have shown that a different fermentative group of hemolytic streptococcus has appeared and finally supplanted the earlier variety. It is suggested that the presence of a second virulent variety may account for the prolonged duration of the disease in these cases.

Except for these two cases, the beta hemolytic streptococcus obtained in subsequent cultures from the ear discharge and from the throat in each individual case belongs to the same fermentative group as the hemolytic streptococcus isolated primarily from that case. Serologic tests carried out on the strains from 9 cases show that all strains isolated originally and subsequently from a single individual case are similar.

In 19 out of 22 cases of bilateral otitis media involved at approximately the same time, the same bacterial species has been obtained in the primary culture from both ears in each individual.

Nine cases of bilateral infections yielding beta hemolytic streptococcus in the primary culture have been studied. In 8 cases, the strains isolated from both ears belong to the same fermentative group, and serologic tests carried out on the strains from 4 cases show that both ears have been infected with the same antigenic variety. One case was investigated in which the beta hemolytic streptococcus found in apparently pure culture in the right ear was of a different fermentative group than that occurring in the left ear. Since the original material was collected from the ear discharges several weeks after spontaneous rupture, no conclusions can be drawn in regard to the primary etiologic organisms.

The strains of beta hemolytic streptococcus from 53 primary ear discharges fall into the following fermentative groups: pyogenes, 45, or 85%; infrequens, 5, or 9.4%; equi, 3 or 5.6%.

The serologic grouping by agglutination and agglutinin absorption tests of the strains of beta hemolytic streptococcus isolated from the primary ear discharge in 44 cases of otitis media show the presence of

3 small serologic groups besides a large number of heterogenous strains, among which no relationships have been found with the 10 antiserums used. The 1st and largest group is made up of the strains from 6 cases; the 2d group consists of the strains from 2 cases, while those from 2 other cases form the 3rd group.

The heterogenicity of the strains isolated from the majority of acute ear infections during the period of increased incidence of respiratory disease in January and February, 1922, indicates the absence of a common primary or secondary causative agent.

From the data available, no deductions can be made concerning the condition of the tonsils in relation to the incidence and later course of the ear infections.

In the cases studied over a considerable period of time, diphtheroid bacilli and staphylococci have been found to be the most frequent secondary invaders in the ear discharge after the acute stage of the infection. There is some indication that in certain cases these bacterial varieties may be factors responsible for the chronicity of the inflammatory process.

The presence of numerous serologic varieties of beta hemolytic streptococci as the apparent etiologic organisms in a series of middle ear infections indicates that the use of a stock streptococcus vaccine is probably of little or no specific value. However, an autogenous vaccine made from the original variety in the ear discharge should be tried.

OCCURRENCE OF BACILLUS BOTULINUS IN HUMAN AND ANIMAL EXCRETA. XXI

E. J. EASTON AND K. F. MEYER

*From the George Williams Hooper Foundation for Medical Research, University of California
Medical School, San Francisco*

*Aided by grants from the National Cannery Association, the Cannery League of California
and the California Olive Association*

In a previous communication,¹ it has been pointed out that *B. botulinus* is probably not disseminated by animal excreta, nor is the intestinal canal the natural habitat of the organism. The presence of *B. botulinus* in over 50% of virgin soil specimens collected in California, suggested more extensive breeding places than the intestines of domesticated or wild animals. In fact, the available information indicated that the organism is capable of multiplying in symbiotic relations with other anaerobes wherever protein material undergoes putrefaction. However, the old theory, which claims that all pathogenic anaerobes are, like *B. tetani*, regular inhabitants of the intestinal canal of animal and man, is so generally accepted that it appeared impossible to refute it without additional systematic studies. Some work along this line has recently been done in China by Carl Tenbroeck and J. H. Bauer.² They tested the feces of 78 patients that were in the hospital for various reasons, and found that 34.7% of them harbored *B. tetani* in the intestinal tract. It is the purpose in this paper to report the findings made on human and animal excreta collected in California during the years 1920-1922. The results confirm the previous conclusions, namely, that animal manure or human excreta is of little or no importance in the distribution of *B. botulinus*.

PREVIOUS FINDINGS OF *B. BOTULINUS* IN EXCRETA

In 1895, van Ermengen³ reported the demonstration of *B. botulinus* in the intestinal contents in a fatal case of botulism, while Meyer and Geiger,⁴ in 1921, demonstrated this organism in the stool specimens in 2 clinical cases on the 6th, 7th and 11th day, respectively, after the consumption of the causative meal. Dickson⁵ isolated *B. botulinus* from the stool of a patient during the same outbreak which supplied one of the fecal specimens used by Meyer and Geiger. Since this publication has appeared, *B. botulinus*, type A, has been

Received for publication, April 8, 1924.

¹ Jour. Infect. Dis., 1922, 31, p. 544.

² Jour. Exper. Med., 1922, 36, p. 261.

³ Centralbl. f. Bakteriol., I, O., 1895, 19, p. 442.

⁴ Pub. Health Rept., 1921, 36, p. 1313.

⁵ Personal communication.

isolated by one of us (K. F. M.) from the stool of a patient on the 6th day of her illness,⁶ and from the colon contents in a case that terminated fatally on the 3rd day after ingestion of the poisonous string beans (unpublished report 103, Healdsburg, Calif.). One fecal specimen procured from a case on the 31st day,⁷ and another from a case on the 26th day of illness⁸ were negative. Graham and Barger⁹ report on the demonstration of this anaerobe, type A, in a specimen of feces and 5 specimens of urine from widely separated spontaneous cases of poliomyelitis in Illinois. Quite recently Tanner and Dack,¹⁰ claim to have demonstrated *B. botulinus*, type B, in 2 of 10 specimens of feces from normal persons. The last named workers employed the plating method in dextrose agar. As this method has been found unsuitable by workers in this laboratory, and the danger of cross contaminations has not been seriously considered in a place where spore resistance tests were being carried out, it appeared advisable to withhold judgment regarding these extraordinary findings until more convincing evidence has been secured.

At frequent intervals, the presence of *B. botulinus* has been reported in the intestinal contents of both healthy and diseased animals. Kempner,¹¹ in 1897, recovered a strain from the intestinal contents of a normal hog, while Burke¹² succeeded in demonstrating the same anaerobe in the manure of a large hog which had recovered from botulism due to home canned peas, 3 months before the sample was taken. Dickson,¹³ who undertook to verify the work of Kempner, examined with negative results the contents of the colon and ileum of 250 grain-fed and 10 garbage-fed hogs. Graham, Brueckner and Pontius¹⁴ isolated *B. botulinus* from the cecum of a horse fatally infected, after drinking water in which contaminated oat hay had been immersed. One of us (K. F. M.) obtained *B. botulinus*, type A, from the colon and intestinal wall of 2 horses from two separate localities. Both animals had died with symptoms of forage poisoning. Similar findings were made on a mule. A report of the isolation of the toxigenic anaerobe, types A and B, from the liver, lymph nodes and intestines of cattle which died in Nevada from the so-called icterohemoglobinuria, have been published by Records and Vawter.¹⁵ Geiger, Dickson and Meyer^{6,7,8} have fully corroborated the findings on specimens secured from similar cases in California and Nevada. Quite recently, *B. botulinus* has been found in the intestines of a normal horse. Three hog specimens examined by Tanner and Dack¹⁵ were found to contain *B. botulinus* type B while three samples from cows gave negative results. The same workers found the spores in one specimen of raw sewage. Dickson,¹⁶ Graham and Scharze¹⁷ Meyer and Geiger⁶ have repeatedly demonstrated *B. botulinus* in the intestinal contents and droppings of chickens that had died of botulism due to home canned vegetables.

It will be noted from this review that, with the exception of the studies by Dickson, and Tanner and Dack, manure specimens from the

⁶ U. S. Public Health Service Pub. Health Bull., 1922, No. 127, Rept. 4.

⁷ Rept. 63 (Reference 6).

⁸ Rept. 81 (Reference 6).

⁹ Univ. Illinois Bull., 1921, 19, No. 7.

¹⁰ Jour. Amer. Med. Assn., 1922, 79, p. 132.

¹¹ Ztschr. f. Hyg. u. Infektionskr., 1897, 26, p. 481.

¹² Jour. Bacteriol., 1919, 4, p. 541.

¹³ Kentucky Agri. Exper. Sta. Bull., No. 207, 1917, p. 92.

¹⁴ Jour. Am. Vet. Assn., 1921, N.S. 60, p. 155.

¹⁵ Jour. Infect. Dis., 1922, 31, p. 92.

¹⁶ Monograph Rockefeller Institute for Medical Research, 1918, No. 8.

¹⁷ Jour. Infect. Dis., 1921, 28, p. 317.

experimentally or accidentally poisoned or infected animals have been tested. In a few instances, namely, in outbreaks of food botulism, it has been possible to test the causative food and establish with considerable accuracy the actual intake of the number of spores, vegetative forms, etc., of *B. botulinus*. On the other hand, however, no attempt has been made to examine the feed or the soil with which the animals supplying the manure specimens came in contact. In the light of previous observations made in this laboratory, and in the course of extensive soil tests which showed a wide distribution of *B. botulinus*, it is quite conceivable that it simply passes through the intestines in spore form without multiplying. A simultaneous examination of the feed and manure of large domestic animals is conducted with considerable difficulty, but in 1920 through the cooperation of Dr. Hayes, University Farm at Davis, Calif., weekly tests on collective specimens of horse, cattle and hog manure, together with the corresponding feeds, have been carried out over a period of two months. Entirely negative findings have been obtained by the procedures customarily employed in this laboratory. Numerous attempts to repeat the same experiments in other regions of the state have failed. It has therefore been decided to examine manure specimens of cattle and hogs from areas in California in which *B. botulinus* is quite common in the soil. The results will be reported in subsequent paragraphs.

In connection with the clinical examination of human stool specimens, Meyer and Geiger⁴ pointed out that the diagnostic value of the demonstration of *B. botulinus* spores could be accepted only when repeated tests on normal stools have established an absence of this organism. A systematic study of the stools of normal human beings who eat raw fruit or vegetables and live in districts in which the organism is quite common in the soil, has been undertaken. Unfortunately, it has been impossible to observe patients convalescing from botulism for a sufficiently long period to decide two important questions: (1) For how many days is a patient with botulism capable of discharging *B. botulinus* spores? (2) Does such a patient remain a spore carrier and as such assist in the progressive pollution of the earth with dangerous bacteria? Being unable to study these two questions, it was therefore necessary for the authors to be content with an examination of 3 series of stool specimens obtained from normal human beings living in 3 different localities of California. The results of 88 examinations are detailed in this paper.

METHOD OF COLLECTION AND EXAMINATION OF MANURE AND STOOL SPECIMENS

Through the cooperation of county farm advisers, health officers and veterinarian, 50 samples of animal excreta were obtained. The manure specimens were collected and shipped in tin containers which were sterilized for 3 hours at 170 C. The human specimens were collected and shipped in sterile 30% glycerol solution and were ground in salt solution and cultivated according to the procedure described in a previous paper.¹⁸ The fecal suspensions were heated for 2 hours at 70 C., mixed with beef-heart peptic digest, covered with a layer of sterile petrolatum and kept at 35 C. for 10 days. Centrifugalized samples of the culture were fed to guinea-pigs, and when found to cause symptoms or death of the animal, adequate specimens were filtered and used in a toxin-antitoxin test. For the latter procedure, mice as well as guinea-pigs were employed. Doubtful as well as negative and positive manure or fecal specimens were cultivated repeatedly for the reasons discussed in a previous paper.¹⁸ Invariably the results of the first culture were confirmed by the 2d or 3rd test.

EXPERIMENTAL DATA

Human Excreta.—Three series of specimens from different parts of California have been examined.

San Francisco: Forty-eight stool specimens secured in June from the healthy personnel of a hospital were cultivated, with negative results. Some of the raw vegetables and fruits served a few days previous to the collection of the samples contained *B. botulinus*, types A and B.

Chico: In Sept. and Oct., through the courtesy of the superintendent of schools, J. B. Hughes, 21 stool specimens were obtained from children living in the rural districts. Previous examination of the soil and vegetables had demonstrated the common and ubiquitous distribution of the spores of *B. botulinus* in this region. The cultures prepared with the specimens were all nontoxic.

Los Angeles County: J. L. Pomeroy, health officer, organized the collection of 19 stool specimens from adults and children (some Mexican) living in the rural districts. In a former paper it has been emphasized that this region, the so-called San Fernando Valley, is heavily infested with the spores of *B. botulinus*, and it can be assumed that the inhabitants of this area ingest these elements quite frequently on raw and poorly washed soil products. The examinations of these specimens were all negative.

The negative findings on 88 stool specimens are definite and conclusive. *B. botulinus* has not been demonstrated in the excreta of healthy normal human beings, although it is known that the spores have been ingested on the raw vegetables and fruit which served as food. It is quite possible that the amount of 100 gm. of feces chosen has been inadequate to demonstrate the few spores present in the sample. Numerous tests with stool specimens artificially contaminated have shown that at least 100 spores must be present to produce a toxic enrichment culture. The results just reported obviously indicate that this number has not been present; in fact, the 88 stool specimens of healthy human beings did not contain as many spores of *B. botulinus* as, for example, a similar amount of soil collected in a region in which the people have been living. The evidence therefore does not encourage the belief that the few ingested spores have multiplied in the intestinal canal and that human excreta used as fertilizer may contribute to the wide distribution of *B. botulinus*. On the other hand, it is recalled that the stool specimens procured from cases of botulism may frequently give positive findings. In the light of the negative results on the speci-

¹⁸ Jour. Infect. Dis., 1922, 31, p. 501.

mens from healthy people, it appears justifiable to conclude that the spores of *B. botulinus* may be demonstrated only in the fecal remnants of the causative food. This view is supported by the negative cultures secured with the feces collected from the cases on the 26th and 31st days of illness, and the absence of the anaerobe in the raw sewage. It is to be regretted that suitable cases have not been available to study the persistence in the intestinal canal of the spores which have been ingested with a causative meal. Experiments conducted on guinea-pigs, rabbits and chickens, and reported in a subsequent paper, definitely indicate that the spores may remain in the intestines for a considerable period of time. The problem of the pathogenicity of *B. botulinus* spores ingested with the food and their behavior in the animal body has already been discussed by Coleman and Meyer,¹⁹ and deserves no further comment.

Animal Excreta.—The findings made on 50 manure samples collected throughout the state of California from hogs, cattle, sheep, horses and chickens are briefly summarized in table 1.

TABLE 1
FINDINGS ON MANURE SAMPLES COLLECTED IN CALIFORNIA

Species of Animal	Total No. Samples Examined	No. of Positive Samples	Type	Origin of Positive Specimen
Garbage fed hogs....	17	3 or 17.6%	3A	(1) Mixed manure of 50 garbage and pasture fed hogs, at Pasadena, Oct. 1921 (2) Mixed manure of 50 garbage, alfalfa and silage fed hogs, Hollister, Nov. 1 (3) Mixed manure of several hundred garbage fed hogs, at Colma, Oct. 1921
Dairy cattle.....	16	2 or 12.5%	2A	(1) Mixed manure of 40 dairy cows, at Stockton, Nov. 1921 (2) Mixed manure of several dairy cows, at North Chico, Oct. 1921
Riding horses.....	10	
Sheep (exper.).....	3	
Chickens.....	4	
Total.....	50	5 or 10%	5A	

The data presented in table 1 corresponds closely with those already reported in a former paper;¹ 10% of the animal excreta contained botulinus spores, while 6.6% of 45 samples of manure gave positive results. In both instances, type A was found. The evidence secured from the examination of 95 manure specimens strongly supports the previous conclusions, namely, that manure or animal excreta contributes relatively little to the pollution of the soil with *B. botulinus*. Hog manure furnished the largest percentage of toxic cultures in this as well as in previous studies. It is quite possible that the intestines of this species are either particularly adapted for the persistence or multiplication of the anaerobe, or the chances for the ingestion of the spores are favored by the nature of the

¹⁹ Jour. Infect. Dis., 1922, 31, p. 622.

feed or the feeding habits of the animal. It is known that the hogs which harbored spores in the intestines were fed on garbage and were pastured on heavily infected soil. The data are in harmony with the previous findings of Kempner, Burke, and Tanner and Dack; but they hardly support the conclusions drawn by recent writers that *B. botulinus* is a normal inhabitant of the intestinal tract of the hog. The same conclusions can be applied to the finding of the anaerobe in the cattle manure.

This investigation, together with other detailed studies conducted in this laboratory and already reported, indicates conclusively that the intestinal canal or the excreta of man or animal play either a negative or subordinate rôle in the multiplication and perpetuation of *B. botulinus*. In the light of the data presented here and elsewhere, it is naturally conceivable that animal manure may quite effectively aid in the country-wide distribution of the highly dangerous heat resistant spores. The same wide dissemination of the spores may also occur on vegetables and fruits transported by trainloads from the West to the East, and as a general rule, from territories where *B. botulinus* spores may be considered most prevalent to those where it is less prevalent.

SUMMARY AND CONCLUSIONS

B. botulinus has not been found in the stools of 83 healthy persons, although it is known that the spores were being ingested on raw fruits and vegetables which served as food.

Three hogs and 2 cattle specimens, or 5 out of 50 samples of animal excreta collected in widely separated areas of California from hogs, cattle, horses, sheep and chickens, contained *B. botulinus*, type A. The evidence secured from an examination of 95 manure specimens strongly indicates that animal excreta contributes relatively little to the pollution of the soil with *B. botulinus*.

GASEOUS METABOLISM OF SOME ANAEROBIC BACTERIA

XIX. METHODS

BELLE G. ANDERSON

From the George Williams Hooper Foundation for Medical Research, University of California Medical School, San Francisco

Aided by grants from the National Cannery Association, the Cannery League of California and the California Olive Assn.

INTRODUCTION

A great deal has been written concerning the gases produced by bacteria of the colon group. T. Smith¹ examined a large number of bacterial strains and classified them in accordance with their quantitative gas analysis. The CO₂ was estimated by filling the culture tube with caustic potash, measuring the diminution in volume; the remainder was combustible, and therefore considered hydrogen. The objections to the Smith fermentation tube for the collection of gases to be subsequently analyzed were summarized by Rogers, Clark, and Davis.² It was pointed out by them that the diffusion of the carbon dioxide from the closed arm of the tube in which the CO₂ tension is high into the area of lower CO₂ tension in the open arm affords considerable opportunity for error. Pennington and Kusel³ were among the first to realize that the methods in practice were not based on a thorough understanding of the principles of the physical properties of gases. They obtained numerous irregularities, and they consequently expressed the view that "until these methods are improved the quantitative chemistry of the living cell cannot progress very far." Harden⁴ appreciated that a correction should be made for both the carbon dioxide dissolved in the medium and that evolved from the excess CaCO₃ by the acids of metabolism. He made this determination in only one sample of medium, but he failed to realize that this correction would vary with each experiment, owing to the fluctuations in the partial pressure of the CO₂. Although the reactions in a medium, kept neutral by an excess of CaCO₃, are different from those occurring with progressive changes of metabolism, he published data with a CO₂/H₂ ratio approximating unity

Received for publication, April 8, 1924.

¹ *Centralbl. f. Bakteriol.*, 1895, 18, p. 1.

² *Jour. Infect. Dis.*, 1914, 14, p. 421.

³ *Jour. Am. Chem. Soc.*, 1900, 22, p. 556.

⁴ *Jour. Chem. Soc.*, 1901, 79, p. 610.

for *B. coli*. His results are fairly comparable to those of later investigators, but the need for different procedures still remained. The early studies were primarily undertaken to obtain data of comparative value for diagnostic work rather than analytical accuracy. Most workers in this field overlooked the fact that each tube examined was subject to different partial pressures of the constituent gases. This condition leads to unequal solubility in the medium and an unequal diffusion of the gases from the fluid. It is now appreciated that in the older methods little of the nearly insoluble hydrogen escaped. The analyses showed obviously an unproportionately large percentage of this gas (90%) in contrast to the amount of the diffusible CO_2 .

The Elredge tube as described by Elredge and Rogers,⁵ used by Ayers, Rupp, and Mudge⁶ in their study of the carbon dioxide production of streptococci, is a marked improvement over the Smith tube. This device permits the use of a small amount of medium (15 c.c.); the gases, which are evolved pass directly into a measured quantity of N/10 $\text{Ba}(\text{OH})_2$. The excess hydroxide is titrated with N/10 oxalic acid, and the CO_2 production is estimated. In experiments dealing with streptococci, the workers used Smith tubes in duplicate; no gas formation was recorded, since this organism forms little insoluble hydrogen. On the other hand, the Elredge tube demonstrated the production of an appreciable amount of carbon dioxide gas. These authors made no attempt to account for the carbon dioxide in the medium. In the Elredge tube, the space above the medium is nearly free from CO_2 , and the low partial pressure decreases the retention of this gas by the fluid which prevents saturation with CO_2 , as occurs in the case of the Smith tube. Their results compare favorably with later work.

J. H. Brown⁷ used a tuberculin syringe as an aspirator to remove the gas from beneath a layer of petrolatum covering the medium in an ordinary test tube. The syringe was also employed as a gas buret for the subsequent analysis. By this method the carbon dioxide production of a large number of strains may be determined with a moderate degree of accuracy and in limited time. The petrolatum tube offers a closed system with a constant volume of medium evolving the gas, instead of a changing volume of fluid as in the Smith tube. The use of the syringe originated from the work of Van Slyke and Stadie⁸ in dealing with the gases of the blood. Brown carried out his gas analyses by drawing the gas and absorbents into the syringe. A determination of the CO_2 in the medium was accomplished by treating the fluid in the same manner as the serum is handled in the ordinary Van Slyke determination for CO_2 . He was able to duplicate his determinations, and secured fairly accurate results by analyzing both medium and gas for carbon dioxide. However, it is evident that he does not absorb the hydrogen and determine the small percentage of nitrogen present. This fraction of nitrogen may be due either to slight denitrification or a possible intake of atmospheric air, while extracting the gas sample from beneath the petrolatum. The H_2/CO_2 ratios obtained by this

⁵ *Centralbl. f. Bakteriol.*, 11, 1914, 40, p. 5.

⁶ *Jour. Infect. Dis.*, 1921, 29, p. 235.

⁷ *Jour. Exper. Med.*, 1922, 35, p. 667.

⁸ *Jour. Biol. Chem.*, 1921, 49, p. 1.

method, which determines the CO_2 volumetrically and takes the hydrogen as a numerical difference, are only approximations of the true values calculated under more exact conditions. Brown made the statement⁹ that "we are able to get almost as accurate results with 0.1 c.c. of gas sample as with 1.0 c.c." The use of such small volumes is certainly undesirable for approximate work even with a syringe graduated to 0.01 c.c. The slightest error in reading such small differences when each 0.01 c.c. division represents 1% of the total volume is magnified greatly by converting them into percentage figures. The method is simple, but economy of either time or medium is not justifiable to the extreme degree of taking 0.1 c.c. gas samples. The sacrifice in accuracy is too great if the data are to be of comparative value in the future or in the hands of other workers. In conclusion, we agree with Brown when he states that his method "retains the simplicity of the Smith tube, at least something of the greater accuracy of the more elaborate methods, and some advantages for the bacteriologist possessed by none of the older methods."

The exact technic of Krogh¹⁰ with a 0.01% accuracy is not necessary in bacteriologic study, while it is essential in the microrespiratory exchange work developed by him. This degree of accuracy far surpasses anything Brown could possibly accomplish, although he uses his maximum amount (1 c.c.) of gas for analysis. Krogh employs volumes from 0.1 to 2.0 c.c. for analysis, using a fine capillary tube for measurements. His micro-analysis method has been tested by Schmit-Jensen;¹¹ the results proved gratifying when additional combustible gases were determined either by combustion or absorption. Krogh (1920) has recently perfected a macrogas analysis apparatus accurate to 0.001%. This degree of accuracy has been attained by paying minute attention to errors due to (1) changes in temperature or pressure, (2) faulty reading of burets when mercury becomes unavoidably oxidized and then adheres to the side of the tube, (3) the varying vapor tensions of the gas absorbents. In order to overcome fluctuations in temperature, he has placed his whole apparatus in a water bath and has utilized a constant pressure compensator. The burets for fine measurements are kept clean by attaching three burets to a manifold; the first, to saturate the sample with water vapor and to approximate the volume of the sample, and the second, to measure the sample accurately. The third is used after all oxygen has been removed before combustion and for subsequent measurements when the gas is free from oxygen. This arrangement and the apparatus described by White¹² represent the best type of equipment obtainable for accurate macrogas analysis at this time.

The type of apparatus selected for a definite bacteriologic problem must be capable of producing data sufficiently accurate to satisfy the purpose of the investigation. After the equipment is selected, consideration must be given to the amounts of gas necessary to operate the apparatus chosen with the least possible error. One of the principles underlying all quantitative analysis depends on the adjustment of the relationship of the magnitude of the unavoidable defects of the process to the size of the sample necessary to make the errors as small as possible. The analysis of gases is subject to greater errors than the ordinary gravimetric processes. In the latter case, at least a 0.2 gm. sample is desirable. A sample of 100 c.c. of CO_2 weighs roughly 0.19 gm. at standard conditions; this size sample is usually advised in commercial analysis, in which the accuracy is 0.1%. While the accuracy of a gas buret depends on

⁹ Jour. Exper. Med., 1922, 35, p. 671.

¹⁰ E. Abderhalden: Handbuch der Biochemischen Arbeitsmethoden, 1915, 8, p. 495.

¹¹ Biochem. Jour., 1920, 14, p. 4.

¹² Technical Gas and Fuel Analysis, 1920, p. 75.

its calibration, there are undoubtedly lower limits to the size of a volume difference which can be read exactly at atmospheric pressures. White,¹² in a modern text on gas analysis, discusses "exact methods" and recommends an apparatus capable of handling a 144 c.c. sample with a side arm 15 c.c. buret, which can be read with an "error of less than 0.02 c.c." The value of an abundant volume of gas and the advisability of a sufficiently large sample to permit duplicate analyses to be made cannot be doubted. A check analysis is always desirable when hydrogen has to be combusted, which combustion necessitates compound measurements instead of absorption in colloidal palladium, as done by Krogh.¹⁰ The residual nitrogen, although a small percentage and totally ignored by some men who used small samples, can be much more readily estimated when using larger amounts of gas. Therefore, it was decided to produce sufficient gas (150 to 200 c.c.) which permits duplicate analyses to be made by means of some common type of gas analysis apparatus accurate to 0.1%. In choosing this course, it is hoped that our experimental conditions might be easily reproduced in other laboratories without difficulty or undue expense.

It is not the purpose of this paper to present a review of the evolution of gas analyses as applied to the field of bacteriology, since the historical aspect previous to 1912 has already been amply reviewed by Keyes.¹³ An extensive bibliography of the subject (up to 1914) is given by Rogers, Clark, and Davis.² However, it is our aim in undertaking analyses of gases produced by anaerobic bacteria, to secure a more exact knowledge of the physiologic processes involved in the changes of the medium subsequent to growth. Much of the data on the differentiation of bacterial species according to gas analyses are either worthless or hopelessly confusing in their contradictory discrepancies, mainly due to the inaccuracies of the technic employed. The only hope of those who wish to see bacteriology become a more systematic science lies in the adoption of the exact methods of the chemist and physicist. This applies mainly to the establishment of the definite physiologic functions involved in bacterial metabolism. Physical as well as chemical methods must be adopted to the specific problem under consideration without making them needlessly cumbersome or adding complicating factors which demonstrate no appreciable increase in accuracy. We heartily agree with Rogers, Clark, and Davis² that workers must decide on a more uniform procedure for bacteriologic gas analyses, if the data thus laboriously accumulated are to be of any intrinsic value as a basis for further research.

The majority of the quantitative investigations of bacterial gases have been carried on with aerobic bacteria, especially the colon-typhoid group. Some studies have also been conducted with soil bacteria important in agriculture on account of their desirable nitrifying properties or their destructive denitrification of the land. The first attempt to analyze the gas of an anaerobic organism was reported in 1897 by Dunham,¹⁴ who isolated several strains of *B. welchii* from fatal wound infections. His work is not of comparative value; he used the Smith tube, which gives a low percentage of CO₂ readings for reasons already discussed. Recently, Wolfe and Harris¹⁵ have made gas analyses in conjunction with their other biochemical determinations on *B. histolyticus*, *B. welchii* (*B. perfringens*), *Vibrio septique*, and *B. sporogenes*, but their results are open to criticism. These workers analyzed only the surface gases, although

¹³ Jour. Med. Res., 1909, 21, p. 69.

¹⁴ Bull. Johns Hopkins Hosp., 1897, 8, p. 68.

¹⁵ Jour. Path. & Bacteriol., 1917, 21, p. 386 (also vols. 22 and 23).

they were evolved in a closed system. On page 418 of their article,¹⁵ when mentioning the gases of *B. welchii*, they state that "on one occasion a mixture containing 90% hydrogen was obtained." In this instance, they were merely analyzing the insoluble surface gas, but the CO₂ in the medium was not estimated. Their statements dealing with the late manifestations of gas production by *B. sporogenes* compared with the more rapid evolution by *B. welchii* are not intrinsic value, since the former organism is essentially a CO₂ producer. Our data to be presented indicate that the sugar in the medium is the source of hydrogen. *B. welchii* is saccharolytic and produces large amounts of hydrogen, while *B. sporogenes* is proteolytic and generates little of the insoluble hydrogen. Not until the medium in which the proteolytic anaerobic *B. sporogenes* is growing has become saturated with CO₂ will the manometer register appreciable gas pressure. This error leads to the false impression that the initial gas evolution is very slow with proteolytic organisms.

Keyes and Gillespie,¹⁶ in studying cultures of *B. welchii*, have employed the exact methods previously described by Keyes.¹³ Several years later Bushnell¹⁷ published some creditable gas analyses in conjunction with other quantitative, biochemical determinations with one strain of the *B. sporogenes* group. These two investigations of the gaseous metabolism of anaerobic bacteria are the only data encountered in the literature which are comparable in degree of accuracy with our results. Rogers, Clark, and Davis² applied their excellent gas analysis methods to the study of *B. coli*, which is primarily an aerobic organism. The possibility of changing the physiologic processes of an organism of essentially aerobic occurrence in nature to anaerobic cultivation is much greater than using strict anaerobiosis with facultative or obligate anaerobes as done in the present study. However, it seems probable that the character of the gas produced has more influence on the growth and metabolism of the organism than the lack of oxygen and the absence of atmospheric pressure.

PERSONAL INVESTIGATIONS

The information to be presented was collected with two purposes in mind; first, the interpretation of anaerobic spoilage produced in canned goods, especially the form attributable to *B. botulinus*; and second, to advance further the biochemical studies on anaerobic fermentation in progress in this laboratory. In order to develop this study it was necessary to establish the constancy of the gas composition for each organism by investigating a number of strains in a uniform medium with the aid of exact apparatus. Furthermore, it seemed desirable to ascertain as fully as possible the mechanism of the gas production by varying the periods of incubation and changing the constituents of the medium. The methods employed are largely those of Rogers, Clark, and Davis,² but a number of modifications have been chosen to suit the particular problem, and some changes have been made which are definite improvements. Keyes¹³ was the first in this country to use a high vacuum

¹⁶ Jour. Biol. Chem., 1913, 13, p. 291.

¹⁷ Jour. Bacteriol., 1922, 7, p. 373.

mercury pump, known as the Toepler pump, to boil the gases from a culture which was grown in a glass bulb closed by two stopcocks. This method is far superior to boiling by means of high temperatures and sweeping the gases into a train of weighed absorbents with neutral nitrogen gas, as done by Stocklasa,¹⁸ when working with nitrification bacteria. Rogers, Clark, and Davis² used the Antroff modification of the Toepler pump, which is better adapted to the purpose of handling large volumes of gas. They improved on Keyes' round glass culture bulb, which has two stopcocks, by using a bulb with glass sealed tips. This avoids the accidental leakage which is apt to occur with stopcocks under high vacuum. The modifications adopted and considered best for our work are herewith described in detail.

Culture Flasks.—The glass-bulbs with two special stopcocks are exceedingly expensive, awkward in handling and untrustworthy when kept under high vacuum for a long period. Keyes mentions no trouble of oxygen leakage; however, his longest period of incubation did not exceed 115 hours. It is not unlikely that the lubricant would give trouble with longer periods of incubation. The bulb used by Rogers, Clark, and Davis,² which is entirely glass sealed, is an improvement, although the shape used by them requires special glass blowing and is difficult to clean. There is no doubt that glass seals present great advantages over stopcocks. They are above suspicion. Consequently, I have made some attempts to use flasks equipped with rubber stoppers carrying glass tubing, which could be sealed off after evacuation. The stoppers were fastened with cements, but the procedure was found unreliable, and therefore it was discontinued. A brief discussion of the method seems, however, justified, in order to deter others from duplicating it.

An Erlenmeyer flask was inoculated and the cotton plug slipped down into the neck in order to permit a sterile rubber stopper to be cemented in place. If the stopper did not fit tightly, the long exposure to high vacuum forced it gradually into the flask, despite the fact that the sides had been shellacked before insertion. Only a round-bottomed flask withstood with certainty the strain of complete vacuum; other types collapsed even after a day in the incubator. First, a commercial casein digest cement, known as "Imperial Cement," was used to seal the stopper, but this preparation, after several days in the incubator, was transformed to a very thin, egg shell-like surface covering, which was not trustworthy. Next a cement composed of 60% shellac and 40% pine tar, which resembles "De Khotinsky cement" in color and hardness, was made. This cement did not dry out at incubator temperatures, but became slightly rubbery due to the oil, so that a nail imprint could be made in it. Gas analyses of culture flasks sealed with both preparations, however, showed the presence of small amounts of oxygen, indicative of leakage. Finally, a hard grade of asphalt, which was very brittle and melted at a high temperature, was used. Although this seal showed none of the visible defects of the others, the metabolic gas showed the presence of oxygen. The asphalt had to be poured on very hot and showed a tendency to bubble. The probable source of trouble lies in the inequality of the coefficients of expansion of the asphalt and the pyrex

¹⁸ *Centralbl. f. Bakteriol.*, 11, 1908, 21, pp. 484 and 620.

glass, leaving space for air seepage, when both are cooled to incubator temperature. The use of paraffin or cements, when the incubation of the culture flasks is done in a water bath as practiced by Wolfe and Harris,¹⁵ has probably not the disadvantages that we have encountered in the incubator.

The culture flask finally selected was a 500 c.c. Kjeldahl with a constricted neck. A round-bottomed glass receptacle of any type will not collapse under vacuum pressure, because the equal angles of incidence of force divide the resultant forces until the pressure is equally distributed throughout the flask, and no compression is possible. The neck of a Kjeldahl was constricted about 3 inches above the flask; a 6-inch piece of pyrex tubing of 1 cm. internal diameter was sealed on at this junction, using the oxygen torch. After plugging the neck with a cotton stopper and dry air sterilization at 175 C. for 3 hours, 300 c.c. of cold medium may be easily poured into the flask. The flasks were autoclaved for half an hour at 15 lbs. pressure. There is no necessity of having two necks, one for introduction of the medium and one for inoculation, as done by Rogers, Clark, and Davis.² They are awkward to manipulate, difficult to repair, and easily broken in washing. Inoculations were made through this long neck; the small tubing was then constricted in the middle by softening it in the blast lamp and pulling it out until a taper was obtained about 3 mm. in diameter at its narrowest point. The cotton plug was then pushed down toward the constriction, and the vacuum tubing of the exhaust pump slipped over the open end. This left the cotton plug protecting the contents of the flask from contamination while exhausting the air, and protecting the oil pump in case the medium suddenly boiled up. After pumping out the air, the flask was infallibly sealed by passing the flame of a hand torch around the constriction. This made a thin tip, which was easily broken in subsequent manipulations. Any round-bottomed flask might be used in place of the pyrex Kjeldahl. If made of soda glass, an oxygen flame would not be necessary, but care must be taken to anneal properly. In this case a large number of Kjeldahl's with broken necks were available.

The size of the flask and the volume of culture medium chosen was very much larger than that used by previous investigators. In numerous instances, rich medium evolved 500-600 c.c. volumes of gas, which were far in excess of that needed for accurate analysis. On the other hand, using less nutrient culture fluids or inhibiting agents such as nitrates or unfermentable sugars, the volume of gas obtained was barely sufficient for a check analysis. One experiment, in which *B. tetani* was grown in 300 c.c. of a 2% peptone and 1% glucose medium for a week, yielded a total gas volume of only 15 c.c. However, since the experimental conditions were standardized for all the data obtained with various organisms and changes of medium, the results are unreservedly comparable.

Culture Fluids.—Although a good deal of work has been done in this laboratory, no synthetic medium in which these anaerobes will really grow has been found. Spores will remain viable or germinate into vegetative forms in mixed inorganic salt solutions. To the best of our knowledge, these organisms need amino acids or protein nitrogen for actual growth. In order to have a homogeneous peptone for all the experiments, 6 pounds of Bacto peptone were pooled and thoroughly mixed. When a medium of a certain percentage was desired, the peptone was weighed out accurately and dissolved in hot tap water; the desired volume made up after the solution had cooled to room temperature. In this way, a plain peptone stock medium was secured, which had quite uniformly a P_H of 7.4-7.6 after sterilization. The 300 c.c. of medium used in each

flask was measured at room temperature, which varied little from 15-20 C. Autoclave sterilization at 15 lbs. pressure for half an hour was considered safer than Arnold sterilization, and apparently did not injure the nutrient properties of the medium. In this connection, it may be stated that the difficulties encountered along the line of contamination with sporulating organisms have been summarized by Dubovsky and Meyer¹⁹ in dealing with the isolation of *B. botulinus*. They say: "If one furthermore appreciates that the spores of *B. botulinus* can survive 4-5 hours boiling and that heated spores may not germinate in from one week to several months, the potential danger of inadequate sterilization is quite evident."

The quantities of various salts or sugars added to each flask of stock peptone solution was sterilized in the Arnold sterilizer for 20 minutes on 3 consecutive days in test tubes. The solutions were prepared so that each tube contained the actual weight of the sugar, salt, etc., dissolved in 10 c.c. of water, necessary to make the percentage finally desired in 300 c.c. of medium. Difco, glucose and Merck's highest purity chemicals were used throughout. All flasks were incubated for 24 hours before they were inoculated with the seed culture.

Seed Cultures.—Stock cultures were kept in beef heart peptic digest liver broth mediums;²⁰ the fluid above the meat was used for the inoculation of the transplants. Medium for making these subcultures were tubed in 10 c.c. quantities covered by a half inch layer of yellow petrolatum. The strains were accustomed to the particular medium chosen for the experiment by growing them for 24 hours in the respective nutrient solution and repeating this process after an incubation of 24 hours. This procedure furnished a vigorous 24-hour culture; 1 c.c. was used to inoculate the gas collection flasks. Observations made in this laboratory by C. C. Dozier indicate that the maximum vegetative growth of *B. botulinus* strains is reached around the 18th hour. By using this method, cultures were conveniently obtained which as a rule exhibited a uniform opacity indicative of a definite number of viable organisms. Taking 1 c.c. of this culture to a flask containing 300 c.c. of medium was considered a sufficiently standardized inoculation. Occasionally, irrespective of the precautions in planting taken to adapt the cultures to the new environment of the experiment, growth in the flasks was absent within 24 hours; this was particularly true when working with plain 2% Difco peptone. In such instances, the flasks were examined in the morning and in the evening; the appearance of the first cloudiness was recorded as the beginning of growth. The majority of flasks showed definite cloudiness after 12 hours' incubation. Analyses made after 3 and 7 days of incubation did not show noteworthy changes in the constituent gases, considering the 4 additional days of growth. Consequently, no concern was felt when the time of visible growth in the flasks with delayed germination could only be approximated within 6 to 12 hours. A delayed germination was encountered only in the less nutrient medium, and for this reason is of little importance with regard to the data to be reported.

The purity of each culture used for analysis was verified by the method recommended by H. H. Heller.²¹ Tubes of a clear, rich liver-peptone agar were boiled for 5 minutes, shaken to remove the air, and allowed to cool to 45 C. Three dilution tubes were made by means of a loop, and the tubes rolled to distribute evenly the inoculum. The colonies of the anaerobes used in this study are characteristic in deep agar shake tubes properly diluted, and con-

¹⁹ Jour. Infect. Dis., 1922, 31, p. 501.

²⁰ Ibid., footnote 17.

²¹ Jour. Bacteriol., 1921, 6, p. 445.

taminations may be detected readily. Shakes have been made from each petrolatum seed culture and also from the flask after growth and evacuation. Gas analyses were made only with and on cultures of established purity.

The Apparatus for Collecting Gas Sample.—The pump shown in fig. 1 is known as the Antropoff modification of the Toepler high vacuum mercury pump. The essential change from the original is the inclination of the mercury chamber *E*. This modification is highly suitable for our work, because it prevents the disastrous pounding of the mercury against the glass which occurs when large volumes of gas bubble through the vertical glass bulb of the original Toepler type. The inclination of the chamber permits the gas to pass smoothly between the upper glass wall and the small vertical but heavy layer of mercury. One whole stroke of the pump can be made at one time without smashing the apparatus. An efficient vacuum of 0.001 mm. has been established for this type of pump by the use of a McLeod gage in the absence of all water vapor. Since the accuracy of a McLeod gage depends entirely on its calibration, and as standardization is impossible in the presence of varying vapor pressures, this type gage previously used by Rogers, Clark, and Davis ² has been replaced by the common U-manometer, marked I in fig. 1. This manometer does not add a volume to the pump system large enough to reduce the pumping efficiency; even under changing conditions it can be accurately read to 0.5 mm. The reading on the manometer indicates roughly the volume of gas in the flask being evacuated. Even a slow leak in any part of the pump is quickly detected by a change in the mercury level of the manometer.

I have chosen the drying bulbs ²² shown in the fig. 1 below the letter *C*, and represents an improvement on the types employed by previous workers. The customary containers for using solid drying agents, such as P_2O_5 or $CaCl_2$, necessitate renewal of the drier as soon as the limited surface becomes moist, caked, and inefficient. In a high vacuum apparatus of this kind which requires absolutely tight seals, such manipulations make the damage due to breakage a serious problem to any one not skilled in glass blowing. The wall of the 150 c.c. bulb coated with crystalline P_2O_5 gives a large drying area, and as the

²² This type of drier *C* is used in the radium emanation pump at the University of California Hospital by the physicist L. B. Clark, who kindly furnished the tube. Over 500 c.c. of loose powdered P_2O_5 are packed into a 3 ft. piece of glass tubing 1 inch in diameter, onto which the bulb is already blown. The end of this long tube is sealed with the torch, and the P_2O_5 slowly sublimed under reduced pressure through the glass wool plug inserted between it and the bulb, using a Langmuir vacuum pump and a hand torch. The vapor condenses in visible crystals on the cold walls of the bulb, and a large and efficient drying surface is obtained. The long tube is sealed off at the desired length (4 inches) below the bulb and forms the trap for catching the liquid phosphoric acid.

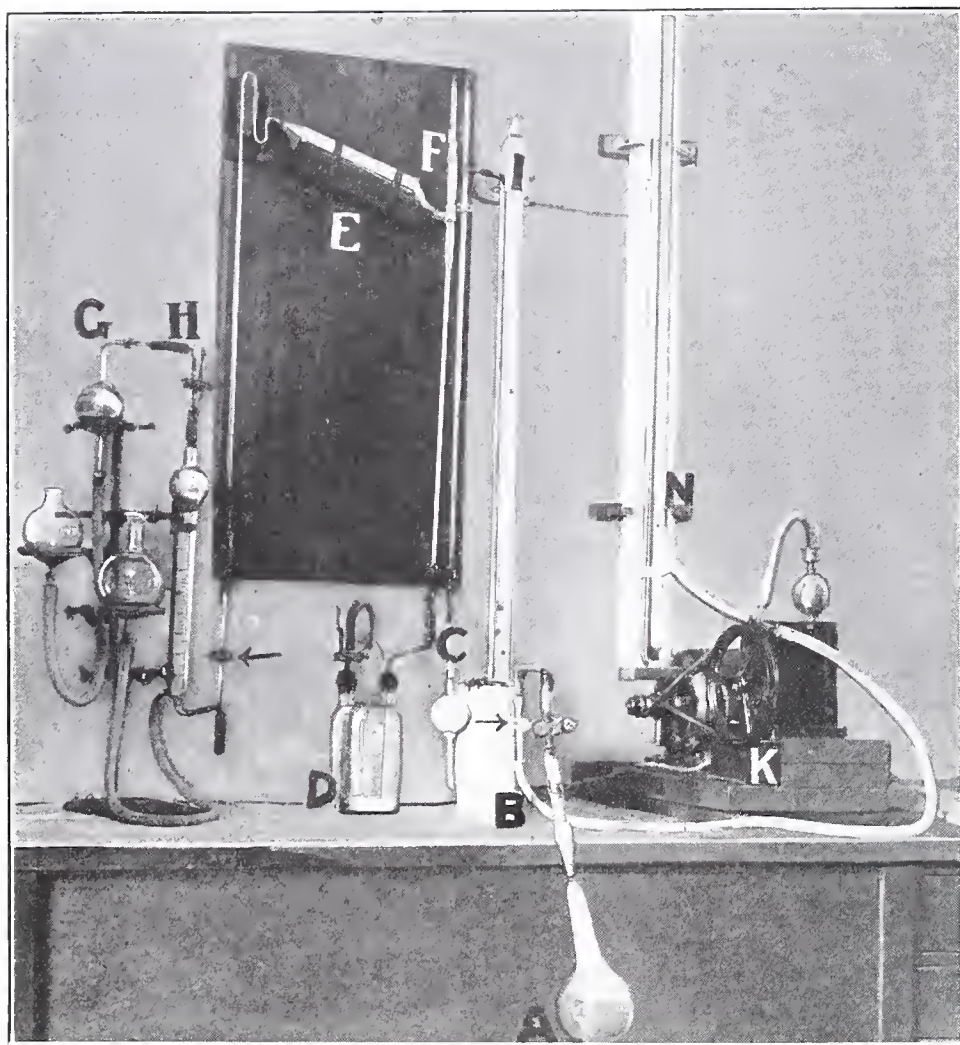


Fig. 1.—Antropoff modification of the Toepler high vacuum mercury pump ready to extract the gas from a bacterial culture. (A) Modified Kjeldahl culture flask attached ready for evacuation. (B) Stopcock connection for preliminary evacuation of system by the oil vacuum pump K. (C) Drying bulb of resublimed, crystalline phosphorous pentoxide (P_2O_5). (D) A two necked Wolfe bottle serves as a mercury reservoir showing connections for using air pressure to raise the mercury. (E) Bulb filled with mercury. (F) Floating valve with spherical ground upper end. (G) Gas sampling bulb with leveling reservoir attached. (H) Eudiometer for collecting and measuring the gas; overflow reservoir attached. (I) U-manometer for recording pressure in the system. (K) Cenco-Hyvac oil pump for preliminary exhaustion of either the Toepler or the evacuation of the culture flasks before incubation. (N) Separate U-manometer connected only to oil pump for use when boiling out culture flasks before inoculation.

liquid phosphoric acid forms, it drains into a trap, leaving an active concave, drying surface. A tube of this kind will need no replacing for 3 or 4 months continuous pumping if the pump is kept closed while not in use, and if care is exercised to prevent the medium in the flask from boiling too violently during evacuation. The sulphuric acid as employed by Rogers, Clark, and Davis is efficient but a slower drier than P_2O_5 . As CO_2 was later found to be soluble in H_2SO_4 , its use was discontinued. Furthermore, the error due to the adsorption of a gas by a solid is infinitely less than the absorption in a liquid such as sulphuric acid. For a time, the P_2O_5 tube was protected by inserting next to the flask a $CaCl_2$ tube, but it was later taken out to increase the efficiency of the pump. The P_2O_5 apparently did not liquefy appreciably faster, consequently the bulb was used unprotected.

About 65% of the gas in the entire system is removed by a single stroke of the pump. The pump is entirely made of pyrex glass, and the volume of the bulb *E* is 600 c.c., which is two thirds of the total volume of the system, including the drying bulb and the space above the medium in flask *A*. The fall tube from *E* to the eudiometer *G* is nearly a meter in length, and must be made with tubing of a millimeter capillary bore. At *F* is a floating valve, which rises with the mercury and prevents the latter from streaming into *C* due to the back pressure of the gas in the upper end of *E*, which becomes great before the gas can be forced over into the eudiometer *H*. This valve has at the upper end a spherical surface well ground to fit the aperture; the hollow valve encloses a small quantity of mercury to pull it down when the mercury level is receding. There are 3 glass feet on the lower surface to prevent the forceful decent of the trap wedging it into the pump arm. The volume of the eudiometer *H* is approximately 350 c.c. and is calibrated into 5 c.c. divisions. The gas sampling bulb *G* will hold about 325 c.c. under compression, and is used to transfer the sample to the apparatus for analysis.

The whole pump as described is mounted on a board, which is securely screwed to the wall. The receptacle *E* is seated in a plaster of Paris mold, which prevents strain of the glass when the large bulb is filled with mercury. Connections leading to the stopcock *B* are likewise set in plaster of Paris. In case a horizontal iron bar rack equipped with metal cups with plaster of Paris molds is available to support absolutely rigidly the great weight of the mercury so that no strain can occur in the glass, the several rubber pressure tubing joints

as shown in the illustration are not necessary. They supply the required elasticity, and when carefully made are absolutely air tight. A pure red gum pressure tubing is used and the bore is moistened with a thick shellac before slipping onto the glass tubing. The ends of the rubber tubing are well sealed with the shellac-tar cement already mentioned previously; it is hard and brittle at room temperatures. If this cement is put on clean, warm glass, satisfactory joints can be made by simply slipping a piece of slightly larger glass tubing over the two ends. Such a joint has been made in attaching the manometer to the main system. The pressure tubing connection just below the stopcock of the eudiometer *H* is found expedient, as this juncture is easily broken when attaching the gas sampling bulb.

The stopcocks needed in the pump are not mercury sealed but the ordinary kind reground with fine emery dust. Mercury sealed stopcocks are expensive and not any more reliable. The lubricant used on these stopcocks is composed of a third by weight of each of pure guttapercha rubber, beeswax, and petrolatum. These constituents are dissolved in benzol. The latter is distilled off at a low temperature under reduced pressure. This process leaves a light brown, tenacious lubricant and prevents carbonization of the rubber. The stopcock just above the flask *A* is needed to prevent the liquid from boiling into the drier when the mercury in the reservoir is receding. It is furthermore useful in locating the source of leakage, whether in the pump itself or at the connection of the flask. The stopcock above *B* (\rightarrow) connects the oil vacuum pump *K* used for the primary evacuation of the Toepler. The exhaust pump (*K*) is known as the Cenco-Hyvac type with a claimed efficiency of 0.001 mm. when filled with fresh oil.²³ This pump has its separate *U*-manometer *N* and is used for pumping out the flasks previous to incubation. The stopcock on the fall tube from *E* is closed when each stroke of the pump is completed. This break in the line prevents the air pressure in the Wolfe bottle *D* from entirely emptying *D* of mercury and letting air rush into the pump when *E* is full of mercury. This occurrence would break the glass when the mercury crashes back against the intersection of *E* and *F*.

Difficulties are encountered in connecting the flask with the pump and in breaking the tip of the flask after complete evacuation of the pump system. The procedure of Rogers, Clark, and Davis² is awkward

²³ Standard Oil Co.'s Zerolene heavy was found as satisfactory as the special oils sold for the same purpose.

and troublesome; it consists of slipping the sealed tip into a constricted glass tube, covering the juncture with ordinary rubber tubing, and then with an inverted rubber nipple filled with mercury. The mercury is easily spilled during the manipulations, but the main trouble is the faulty breaking of the glassware under the joint. Although the tip is always scratched with a file, the leverage from this tip to the rim of the encasing constricted heavier pyrex tube, a distance of about an inch, chipped large pieces out of the encasing rim and caused leakage. A large stopcock with an 8 mm. bore has subsequently been used. The tip is placed inside the cock, and the scratched taper is quite readily broken by turning the stopcock with steady pressure. This procedure in opening the flasks is more satisfactory, and although an inch of tubing is left below the stopcock, the leverage is often great enough to break the lower rim badly, and samples may be lost in this manner. The large stopcocks are made only in soda glass, which is so brittle that after several usages nicks are made on the rim of the bore of the solid cock, causing it to leak. The use of a hot platinum wire sealed into the connection to break the tip is not dependable. The glass frequently cracks where the wire is sealed into it. The next procedure consists of working the tip into a 3-inch piece of pressure tubing and breaking the tip by pressure with the fingers. If a clean break is made, the high vacuum causes the tubing to form a tight seal around the broken tip, and the gases present in the flask cannot enter the main pump. On the other hand, if this tip is crushed with forceps, the sharp glass cuts the tubing, and after several such openings definite leakage has been observed.

The connection finally adopted is simple and nonbreakable. A piece of brass metal tubing, 3 cm. long and 1 cm. in diameter, is worked into the middle of a piece of pressure tubing, 12 cm. long with an 8 mm. bore. This rubber connection is attached to the stopcock above *A*, and the neck of the flask is fitted until the tip is within the metal rim. The lower end of the rubber is wired to the flask, and a plastic modeling clay, known as "plasticine,"²⁴ is molded over the joint to insure a tight seal. It is better than a brittle cement which cracks and leaks when the flask *A* is shaken to liberate completely the gas in the medium. After the entire pump is air tight, the tip is easily broken by pressing it against the metal tube. This rigid core prevents the rubber tubing

²⁴ Plasticine is very fine grained, contains some oil, and can be obtained in this country in large 8 inch sticks; it is made by Harbutt Co., Bath, England.

from collapsing, keeping the passage between the flask and the pump open for transferring the gas. This connection is simple in operation and reliable, as proved by the absence of oxygen in the analysis of the gas.

Manipulations.—The culture flask already described is inoculated from the petrolatum tube transplant, and agar shakes are prepared from the seed tube. The neck of the flask is then constricted in the middle by heating it in the blast lamp. The flask is evacuated by the pump *K* until it boils vigorously, and all the air is expelled by shaking the medium while the pump is going. After pumping from 10 to 15 minutes in order to flush out the air by the water vapor, the constriction is sealed by means of a hand torch. The flask is then ready for incubation. After growth has taken place, the flask is cooled to room temperature before pumping out the gas, in order to protect the drier from excessive water vapors.

When the incubation is completed, the flask is connected to the Toepler in the manner previously described, and the system is evacuated by the pump *K* through the stopcock *B*. After closing *B*, the manometer is read. A second reading is made after an elapse of 10 minutes, to ascertain that no leaks have occurred. The stopcock above *A* when closed will help to locate any leak; the stopcocks are regreased if necessary. When everything is tight, the mercury in the reservoir *D* is forced into *E* by air pressure admitted through the three-way stopcock on *D*. The valve *F* must be watched as it may not rise rapidly enough to prevent the stream of mercury from falling into *C*. A small bubble of air is transferred to *H* from the fall tube, and the stopcock on the latter is closed so that no more mercury will be forced over. The pressure in the reservoir is then slowly released by opening the three-way stopcock on *D*. The pressure must not be released too rapidly, especially if there is considerable gas in the flask, which causes the trap to descend forcefully and wedges it into the aperture leading to the pump, which has a total vacuum. In case the gas rushes into *E* the mercury will crash back against the glass and break it. Should the trap stick, it may be tapped with a strip of rubber to loosen it and cause its easy and smooth descent. Every trace of air must be pumped out before the tip on the flask *A* is broken. Observation of the manometer, the sharp click of the mercury at the upper end of *E* and the transference of no gas into *G* indicates the perfection of a total vacuum. Care must be exercised not to force the mercury violently against the capillary tubing joint at the upper end of *E* when a complete vacuum has been attained.

The tip of the flask is broken by pressing it against the metal rim in the rubber connection. The flask is carefully shaken before recording the pressure caused in the system by the release of the gas. The stopcock above *A* is now closed and a minute allowed for the drier to act before making a stroke of the pump. The eudiometer *H*, the bulb *G* and all connections are filled with mercury. Pumping is begun and the gas forced from *E* into *H*. The stopcock above *A* is opened to let in more gas from the flask as soon as the mercury has receded into *D*. Pumping is continued until a constant boiling point of the solution is reached, as indicated by the manometer and by the negligible quantities of gas transferred by each stroke. The gas is easily measured in *H* and run into *G*, which with its leveling bulb can be disconnected and taken to the analyzing apparatus. A hydrogen-ion determination is now made with a portion of the contents of the flask, and agar shakes are prepared to test the cultures for purity.

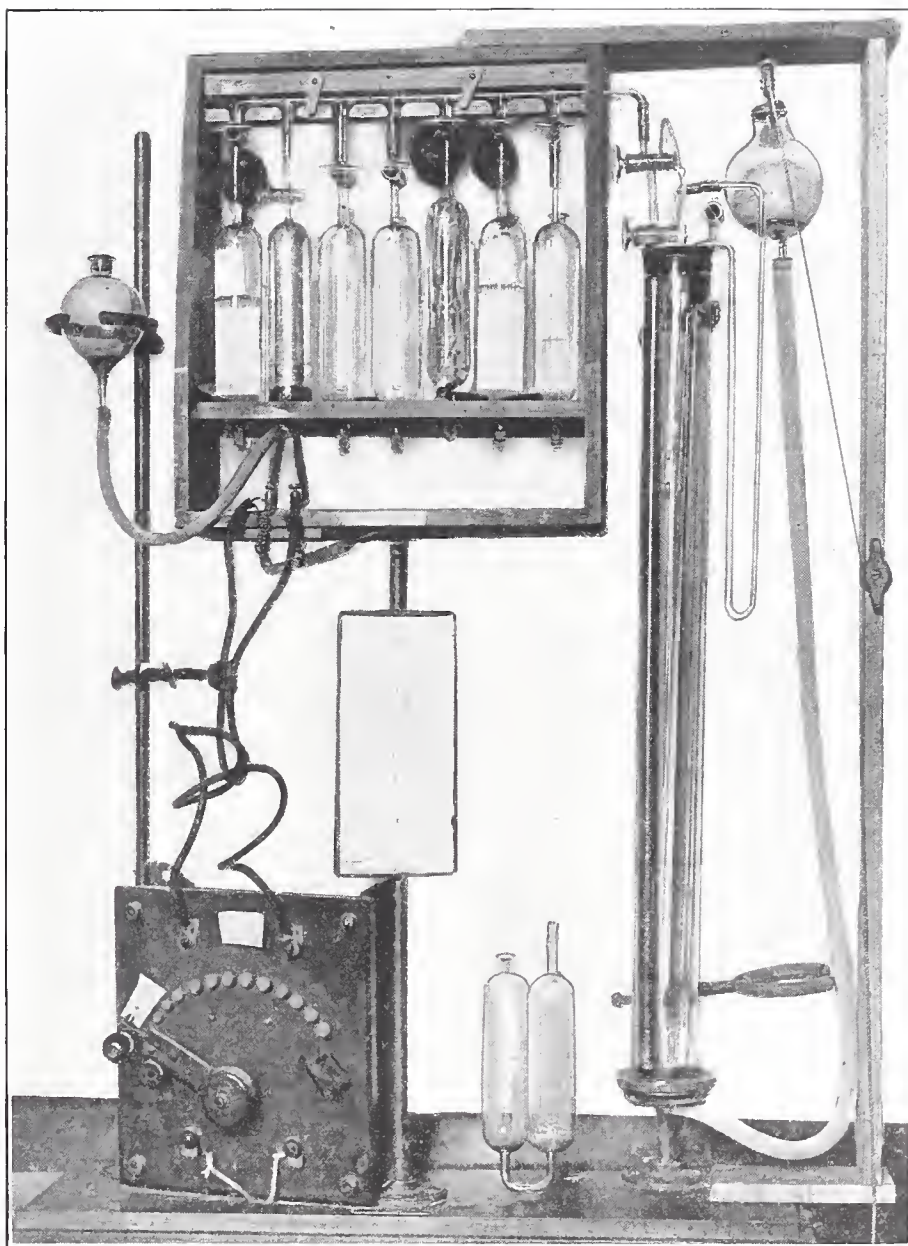


Fig. 2.—Burrell gas analysis apparatus, showing the type of pipet, controlling rheostat, calibration curve for buret, and pulley attachment for working the leveling bulb.

The Gas Analysis Apparatus.—The Burrell modification of the Orsat apparatus illustrated in fig. 2 has been used instead of the Hempel or Haldane type commonly employed in biologic work. A detailed diagrammatic explanation of the apparatus has been published by the U. S. Bureau of Mines.²⁵ It has a 100 c.c. buret graduated in 0.1 c.c. divisions, allowing a reading to be made within 0.05 c.c. The undesirable rubber connections between absorbing pipets and leveling bulbs as used in the Haldane are replaced by a type of pipet which is entirely made of glass. The stationary train of pipets when carefully operated is preferable to the continual changing of separate pipets as done in Hempel's method. The buret was calibrated by weighing exactly every 5 c.c. of mercury discharged and making the calibration curve as shown in fig. 2. Analyses secured with this apparatus using moderately large samples are accurate to 0.1%. Most of the determinations were done in duplicate, mainly to verify the results of the combustion analysis. Usually the sample taken varied between 75-100 c.c.; a film of dilute nitric acid was kept on the mercury in the buret to saturate the gas before it was measured.

For ordinary work, a train of 7 pipets is unnecessary, but they proved useful when a gas of questionable fractionation was studied. A wooden extension supporting a pulley was placed on the right side of the pipet rack. With this improvement, the heavy mercury leveling bulb attached to the buret can be handled with less fatigue to the operator. Mercury, which is usually employed in the manometer to equalize the pressure in the buret with that in the compensator before making a reading, is too heavy and not sufficiently sensitive to slight pressure. Water, on the other hand, is so easily forced over into the compensator²⁶ that an acid water solution of 5% glycerol colored with methyl orange has been used.

A side view of a pipet is illustrated in fig. 2 on the base stand. The distended rubber toy balloons seen behind the manifold stopcocks protect the solutions. The controlling stop-rheostat shown on the board controls the glow in a 26 gage platinum spiral in a combustion pipet. The 110 volt a.c. current lighting circuit serves as a source of electricity.

The CO₂ absorbent is a 10% potassium hydroxide solution; this strength works rapidly and efficiently and does not lower the vapor pressure in the gas to the same degree as a stronger preparation. For some work a known strength of Ba(OH)₂ has been used in the bubbling

²⁵ Burrell and Siebert: U. S. Bureau of Mines, 1913, Bull. No. 42, p. 43.

²⁶ The compensator may be improved by a stopcock at its base to facilitate its drainage in case the manometer contents is forced over.

pipets Nos. 4 and 5 counted from the buret. The excess hydroxide has been titrated with oxalic acid. This procedure has been chosen in order to determine the CO_2 titrametrically. From the volumetric difference, the percentage of extraneous gas absorbed in this fraction is calculated. Vertical sticks of yellow phosphorus in water in a special pipet (No. 3) is used for oxygen absorption, and found less troublesome than an alkaline pyrogallic acid solution. The phosphorus pipet has remained active for a year or more; it is covered with black paper, when not in use. These absorbants are well saturated with the gas to be studied before beginning the analysis. The combustion pipet, as shown in fig. 2, is filled with mercury. Pure oxygen gives violent explosions with rich mixtures of hydrogen and favors the oxidation of the residual nitrogen. Air is used as the source of oxygen for combustion.

INVESTIGATION OF THE CONSTITUENT GASES

It is generally assumed that the gases formed in the course of a bacterial fermentation consist mainly of CO_2 , H_2 and N_2 . However, it must be recalled that a number of investigators reported the finding of CH_4 , CO , and N_2O and other oxides of nitrogen in the gases produced by aerobic bacteria or soil organisms. The publications of Tacke,²⁷ Hesse,²⁸ Pfeiffer and Lemmerman,²⁹ and others, dealing with these rarer gases, were practically all published before 1900. A careful perusal leaves no doubt that the observations are probably incorrect due to inadequate methods of analysis. Apparently no attempt has been made to calculate the amount or to fractionate the odorous constituents of the gas of anaerobic growth. The literature has been searched to ascertain the amines, alcohols, mercaptans, etc., which assume the gaseous state at ordinary temperatures, and have been demonstrated as products of bacterial metabolism. In the study under consideration, hydrogen sulphide has been observed qualitatively in the gases. An extensive research has been undertaken to adapt some of the methods to the quantitative determination of oxides of nitrogen and H_2S in the gases formed by *B. botulinus*, *B. sporogenes* and *B. welchii*. A brief account of the procedures which have been employed to determine the rarer constituents is herewith given.

²⁷ Landwirtsch. Jahrbücher, 1887, 16, p. 321.

²⁸ Ztschr. f. Hyg. u. Infektionskr., 1893, 15, pp. 24 and 183.

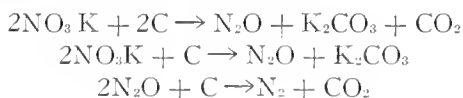
²⁹ Landwirtsch. Versuchsstationen, 1898, 50, pp. 115 and 143.

Methane.—This gas forms CO_2 when burned with air in the combustion pipet. However, no CO_2 was demonstrated after combustion in any gas sample carefully analyzed. Therefore it is concluded that CH_4 or other carbonaceous gases are not metabolic products of the 3 species of anaerobic bacteria investigated.

Carbon Monoxide.—An ammoniacal cuprous chloride absorbent for CO was used in a bubbling pipet for numerous analyses. No absorption took place. The lack of CO_2 formation in the combustion operation, already referred to, confirms the absence of CO gas.

Gaseous Ammonia.—The hydrogen-ion concentration of the culture is so acid that no NH_3 gas would be released. All NH_3 formed during growth is retained in the medium in the form of stable acid salts.

Oxides of Nitrogen.—The presence of residual nitrogen in the analyses indicates that slight denitrification is taking place. Beijerinck and Minkman³⁰ worked extensively on the formation and decomposition of oxides of nitrogen by soil bacteria. They find a high percentage of N_2O as an intermediate product of denitrification. They explain this formation in the following manner:



These workers consider the ammonia salts which result from protein and amino-nitrogen metabolism as another source of the nitrogen found. They fail to give a detailed account of their hydrogen combustion technic, but their high percentage of oxides of nitrogen have thus far not been confirmed. However, additional investigations of the probable source of the nitrogen found in the gases is deemed necessary.

Insoluble N_2O passes through all absorbents into the combustion pipet. In the presence of hydrogen, the following reaction occurs when the platinum spiral is heated before air is admitted:



This operation is done repeatedly before making the hydrogen determination. No contraction in volume occurs; it is concluded that no N_2O is present. All other oxides of nitrogen are soluble in the first pipet containing alkali. Consequently, methods have been devised to analyze the KOH solution in the pipet for the presence of nitrates.

The Bureau of Mines³¹ has adapted for detecting oxides of nitrogen in mine air the colorimetric method for nitrates used in the public health water analysis. This method depends on the intensity of the yellow color of tri-potassium nitrodisulphonate formed from phenoldisulphonic acid in the presence of potassium nitrate. The technic was as follows: A 100 c.c. sample of gas was absorbed in 200 c.c. of N/50 KOH contained in the ordinary gas absorption pipet. Any oxides of nitrogen in this solution were then oxidized to nitrates by adding 5 c.c. of 30% hydrogen peroxide and evaporating the solution to dryness on a hot plate previous to the treatment with phenoldisulphonic acid. This method is delicate, and nitrates are reported as parts per million. The original KOH solution had to be diluted before adding the phenoldisulphonic

³⁰ Centralbl. f. Bakteriöl., II, 1910, 25, p. 30.

³¹ Allison, Parker and Jones: U. S. Bureau of Mines, Tech. paper No. 249 (1921).

acid in order to read accurately the intensity of the yellow color. This determination was made on gases from cultures of *B. botulinus*, *B. sporogenes* and *B. welchii*. Nitrates varying in amount from 0.2%-0.4% were formed. These findings are interesting, but in an 85% CO₂ fraction they constitute a negligible percentage of error. Control determinations were made on the distilled water and the hydroxide, but no appreciable color was secured. There was a slight brownish yellow shade in the 100 c. c. Nessler tubes which contained the unknown nitrate. The brown tinge is likely to lead to erroneously high values. The color indicates that either some organic gas was present or that the large amount of CO₂ interfered with the reaction. Subsequently, some acetic acid was added to boil off the CO₂ from any K₂CO₃ in the salt mixture before adding the phenol-disulphonic acid. Other errors inherent to this method as applied to water analysis are discussed at length by Chamott, Pratt, and Redfield.³² The percentage obtained is such a small error in the larger CO₂ fraction that no further determinations of the oxides of nitrogen appeared justified.

The gases of the odorous fraction were now investigated. The rank odor of gas, as well as the CO₂, is absorbed by the KOH pipet. The alkaline solution acquires an odor resembling spoiled cabbage. All gases of an acid reaction, e. g., CO₂ and H₂S, are removed by the KOH solution. Alkaline gases, if they are very soluble, are removed by the first absorber, and in this particular instance they increase the error in the CO₂ fraction. A brief speculation on the probable constituents composing this unknown fraction seems appropriate in this connection.

Volatile Amines.—There is little said in the literature regarding the quantitative estimation of organic sulphur gases or volatile amines in bacterial metabolic products. In view of this fact, it appeared unwise to undertake a study of these fractions. The quantitative determination of amines even in the medium in the presence of ammonia salts is unreliable. A number of amines have been qualitatively demonstrated in the decomposition products of many bacteria. M. Guggenheim³³ in his excellent treatise on the "Proteinogenous Amines" has emphasized the widespread occurrence of amines due to bacterial activity. Paul Hirsh³⁴ discusses from a different standpoint the effects of bacterial growth on the protein bodies and the amines commonly encountered. After reviewing these detailed and complete monographs, it appeared as quite likely that odorous products such as CH₃NH₂ b. p., 6.5°C.; (CH₃)₂ NH b. p., 7.2°C.; and (CH₃)₃ N b. p., 3.2°C., are formed in cultures of the anaerobes.³⁵ The volatility converts these liquid amines into gases at ordinary temperatures, and as such form at least a part of the disagreeable odor. Slight amounts of higher amines, such as putrescine and cadaverine (pentamethylene-diamines), are to be expected in any prolonged protein decomposition, but it is impossible to demonstrate them in small quantities. These amines resemble ammonia in alkaline properties and marked solubility; they are absorbed in the first pipet in any analysis.

Mercaptans and Thioethers.—The boiling points of a few of these organic sulphur compounds are now considered; they are briefly as follows: Methyl sulphide (CH₃)₂ S b. p. 38°C.; ethyl sulphide (C₂H₅)₂ S b. p. 91°C.; methyl mercaptan CH₃SH b. p. 6°C.; ethyl mercaptan C₂H₅SH b. p. 36°C. They are neutral compounds, insoluble in water, yield metallic salts, and have exceed-

³² Jour. Am. Chem. Soc., 1911, 33, p. 336.

³³ Die Biogenen Amine, 1920.

³⁴ Die Einwirkung von Mikroorganismen auf die Eiweisskörper, 1918.

³⁵ b.p., abbreviation for boiling point.

ingly foul odors. It is evident that most of them are found in the gaseous state at ordinary temperatures. The formulas show plainly that they are derived directly from their corresponding alcohols or alcoholic esters. L. Mathieu³⁶ discusses the formation of compounds of this type during alcoholic fermentation. He emphasizes the fact that the formation of alcoholic compounds is as common in bacterial metabolism as H_2S production. Alcohol formation by many anaerobic bacterial species has been fully established in this laboratory. This fact combined with definite proof of H_2S formation in the cultures studied supports the belief that some of the lower alkyl sulphides (thioethers) and mercaptans are either synthesized or found as primary split products.

Sasaki and Otsuka³⁷ have worked with aerobes grown on medium containing cystin, sulphates, sulphur and taurin. They found no mercaptan formation with pure cultures. The technic of the Denigésscher reaction employed by them as a test for mercaptans could not be repeated, because no mention of this reaction was located in the literature. Herter³⁸ uses isatin in concentrated sulphuric acid solution for a qualitative mercaptan test; the reaction is considered positive when the color changes from red to different shades of olive green. The expensive isatin was not available, and the uncertainty of the methods thus far described did not warrant detailed research along this line.

The difficult estimation of such organic compounds as mercaptans is demonstrated by the work of Nencki and Sieber.³⁹ Their medium consisted of 600 gm. of meat in 3 liters of water. After 45 days' incubation with *B. liquefaciens magnus* (?), they acidified the whole culture with oxalic acid, and distilled as completely as possible the odorous gaseous products into a 3% $HgCN$ solution. They obtained an amorphous mass with a few leaflike yellow-green crystals, but they could not isolate the mercury mercaptan. They tried to redistill this precipitate from concentrated nitric acid into 10% $PbAc$ solution. The yellow lead precipitate showed microscopically beautiful prismatic crystals. The dried crystals weighed 0.142 gm. and yielded 0.1426 gm. of $PbSO_4$, which corresponds to 68.5% of lead in the original sample. This value approximates closely the percentage of lead in the formula $(CH_3S)_2Pb$ of the probable mercaptan, which is 68.76% lead. This yield indicates the formation of about a decigram of mercaptan in a large volume of a rich meat infusion medium after an incubation of 45 days. On the other hand, they claim that the olfactory senses of man can detect 1/460 millionth of a mg. of mercaptan. The minute yield of this substance from a small volume of less nutrient medium incubated for a short period would unquestionably be sufficient to confer to the metabolic gases an offensive smell. However, the quantitative determination of such small amounts of mercaptan from either culture or gas, even using the most up-to-date methods, would be highly impractical and impossible. Furthermore, the foul odor characteristic of anaerobic gases is probably due to a combination of individually rank smelling compounds previously mentioned, e. g. amines, mercaptans, thioethers and hydrogen sulphide.

Quantitative Hydrogen Sulphide Determinations.—The most promising outlook to determine the source of the odorous gas was offered by the quantitative estimation of the gaseous H_2S evolved. The analytical

³⁶ Chem. Centralbl., 1911, 2, p. 1256.

³⁷ Biochem. Zschr., 1912, 39, p. 208.

³⁸ Jour. Biol. Chem., 1905, 1, p. 421.

³⁹ Monatshefte f. Chemie, 1889, 10, p. 526.

research to be reported involved the following procedures: (a) determination of the sulphur metabolized by the organisms; (b) direct quantitative absorption of the H_2S in the gas; (c) titration of the absorbant containing H_2S ; (d) gravimetric analysis of absorbant containing H_2S , and (e) exact titration of the CO_2 , estimating the H_2S as the remainder of the alkali soluble fraction.

(a) *Gravimetric Analyses for Sulphur Lost by the Medium.*—Sulphur determinations on the culture medium were made by combining the method which Redfield and Huckle⁴⁰ used on peptone and that which was employed by Denis⁴¹ in working with urines. To 100 c.c. of medium contained in a deep 3 inch porcelain dish, 20 c.c. of concentrated HNO_3 and 5 c.c. of modified Denis copper reagent was added; the whole evaporated to dryness on the steam bath. The copper reagent was composed of 25 gm. each of $\text{Cu}(\text{NO}_3)_2$ and NaCl in 100 c.c. of water. The NH_3NO_3 used by Denis was omitted, because it favored spattering during combustion. The nitrate oxidation was given preference to the alkali digestion employed by Redfield and Huckle, as the latter demands the continual attention of the operator. These authors found no appreciable loss of sulphur in an open dish digestion for several hours with HNO_3 . They closely duplicated their results by sealed tube digestion for 2 days. The nitrate salt mixture was thoroughly dried in a hot air oven. It was then combusted slowly to red heat in an electric furnace. The combustion must be gradual, or the total carbon content will not be entirely oxidized but charred. The furnace was heated through 5 stops, changing the temperature slowly from 190 C. to 385 C. As a rule, it took nearly one entire afternoon to complete the combustion; however, a number of samples could be run at one time, as they required little personal attention. The black mixture of oxides and sulphates was dissolved in concentrated HCl , diluted and neutralized with NaOH . Sufficient dilute HCl was added barely to keep the $\text{Cu}(\text{OH})_2$ in solution. The sulphate was precipitated from a clear blue faintly acid solution with 10% BaCl_2 in the usual manner. The BaSO_4 obtained by the procedure described and the amount precipitated in duplicate determinations by the technic of Redfield and Huckle agreed within 2 mg. in a 302 mg. precipitate, which

⁴⁰ Jour. Am. Chem. Soc., 1915, 37, p. 607.

⁴¹ Jour. Biol. Chem., 1910, 8, p. 401.

may be considered a negligible error. According to personal observations, the sulphur analysis gave very good checks on duplicate flasks of medium.

(b) *Direct Quantitative Absorption of H_2S .*—In the light of the work of Klein,⁴² the use of organic liquid reagents for absorption did not look promising. This worker found that many gases will react in various liquids when a trace of moisture is present; the same occurs in other absorbants even when carefully dried. The use of such absorbing liquids as carbon bisulphide, ether and benzol was impossible on account of the difficulty of drying thoroughly, both absorbent and gas.

The Bureau of Mines has employed acetates of lead and cadmium in various ways for estimating H_2S . Consequently, absorbing solutions of both these salts in 10% H_2SO_4 were tried in the pipets. Slight precipitates of characteristic color were secured in both cases, but they soon broke up into invisible particles after further agitation of the absorbent. Dupasquier's method mentioned by Dennis⁴³ was tried, absorbing the gas in N/20 iodine according to the reaction— $H_2S + 2I \rightarrow 2HI + S$. Small particles of sulphur appeared on the surface of the iodine. Solutions of the character mentioned in the foregoing paragraph failed to give a sharp constant volume end point after 3-5 minutes' agitation, indicating a diffusion of other gases (CO_2) into the absorbant. For this reason, the use of such aqueous solutions of inert salts for direct volumetric absorption were abandoned.

The statement made by White⁴⁴ suggested another absorbing solution. He stated, when dealing with the exact analysis of CO_2 : "If other acid gases such as H_2S , SO_2 or HCl are present they may be removed by first shaking the gas in a pipet containing $KMnO_4$ faintly acidified with H_2SO_4 ." A 1% solution of $KMnO_4$ was made and 2% H_2SO_4 added; this degree of acidity does not liberate oxygen from the permanganate. The initial absorption of gas was more rapid by this reagent than by the solutions previously considered. However, it continued to absorb the residual gas slowly. This occurrence led to an increase of the acidity in order to decrease the solubility of the CO_2 in the absorbant. It was found that more than 5% acid decomposed the $KMnO_4$, liberating oxygen. An acid solution of this strength was

⁴² Jour. Phys. Chem., 1911, 15, p. 1.

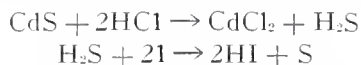
⁴³ Gas Analysis, 1913, p. 272.

⁴⁴ Technical Gas and Fuel Analysis, 1920, p. 84.

as unsatisfactory as a lower acid concentration for obtaining a constant volume reading. Curiosity prompted the filling of a pipet with concentrated H_2SO_4 ; it was found that from 10% to 15% of commercial CO_2 was soluble in a relatively short time. This finding demonstrates clearly the futility of employing acid reagents for quantitative volumetric absorption as recommended by such reputable authors as Dennis and White for removing contaminating gases without changing the CO_2 fraction.

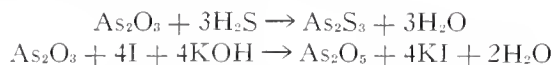
From the observation just recorded, it was reasoned that a solution of acid KMnO_4 already saturated with commercial CO_2 should permit the CO_2 of the gas sample to pass unaltered quantitatively through it. By passing the CO_2 until no diminution in volume took place a saturated solution was obtained. This procedure, however, gave a supersaturated solution when a bacterial gas sample was analyzed. A considerable pressure of CO_2 had evidently collected over the solution in the rear reservoir retained by the balloon. During analysis, the excess CO_2 in the rear reservoir diffused through the solution in order to equalize the partial pressure of CO_2 in the gas sample of the pipet bulb. This difficulty of super saturation was remedied by arranging a leveling reservoir of CO_2 , which was attached to the rear bulb of the pipet. The first flask of the device contained CO_2 over acid water, and the second served by means of a syphon as a compensator for the changing volumes during agitation. In this manner it was anticipated that a solution saturated at atmospheric pressure without undesirable diffusion from the rear bulb of the pipet could be obtained. A plain pipet without the vertical glass tubes was used. As the thin film of absorbent on the vertical tubes of the ordinary absorbing pipets created an unstable gas equilibrium, the less agitated surface of a liquid in a plain pipet was chosen. A small percentage of the gas was readily soluble in this saturated reagent. A number of samples were analyzed in this manner, and the CO_2 fraction estimated by direct absorption in hydroxide. This technic was more dependable than any of the others previously tried. The end point of the absorption was not sufficiently clean-cut before the partial pressures of the CO_2 in the sample and the partial pressure of CO_2 content of the absorbent began to equalize. In view of the fact that the CO_2 percentage of the samples ranged from 50% to 97%, this equilization of partial pressure sometimes meant an absorption of CO_2 from the sample, or at other times a liberation of CO_2 from the liquid. At best these determinations would be only approximate; consequently this procedure was discontinued and other methods investigated.

(c) *Titration of Absorbent Containing H_2S .*—An ammonical $CdCl_2$ solution was used in a bubbling pipet as suggested by White;⁴⁵ titrating the CdS with iodine according to the following reactions:



The entire contents of the pipet were titrated requiring 2.1 c.c. of N/10 iodine. The CdS precipitate from the same gas sample was filtered from another absorbing pipet, dissolved in dilute HCl , and when the precipitate was titrated with N/10 iodine only 0.5 c.c. iodine was required. This variation in titration value indicated that the ammonium chloride in the previous titration must have influenced the reaction. In order to check the titration values, sulphur analyses were made on the medium before inoculation and after evacuation. In this instance it was calculated that the total amount of sulphur metabolized if converted into H_2S would equal 2.83% of the total gas. In comparison to this figure, the highest titration value would give 5.2% H_2S and the lowest 1.25% of H_2S . The amount of N/10 iodine which reacted in either case was not large enough to be reliable for a quantitative estimation.

Another titratmetric method has been published by Sutton.⁴⁶ White⁴⁷ also mentions the use of arsenious acid for H_2S determinations. Acid As_2O_3 reacts with H_2S and the excess is titrated with N/100 iodine according to the following reactions:



The percentage of H_2S calculated by this process was 10%, which was utterly impossible. Later the statement in Sutton⁴⁸ was found that in the presence of alkali bicarbonates the reaction gives "defective results with bleached analysis from some cause not yet understood." Undoubtedly the CO_2 dissolved produces some bicarbonates which cause the iodine titration to bleach incorrectly.

The next titration method which was used, depended on the titration of soluble K_2S with iodine suggested by Sutton.⁴⁹ He states that "Sulfurated hydrogen in mineral waters may be accurately determined

⁴⁵ Ibid., p. 169.

⁴⁶ Volumetric Analysis, 1911, p. 347.

⁴⁷ Technical Gas and Fuel Analysis, p. 72.

⁴⁸ Volumetric Analysis, p. 139, footnote.

⁴⁹ Ibid., p. 348.

by iodine." It has been shown by Brunck⁵⁰ that an iodine solution added to a neutral alkali sulphide solution gives low and variable results. But an accurate titration can be made by slowly adding the sulphide to approximately the theoretical amount of slightly acid iodine solution, stirring rapidly. An investigation of the titration of sulphurous acid and sulphite solutions with iodine has led Raschig⁵¹ to a similar conclusion; namely, consistent results can be obtained by adding the sulphite solution to the iodine solution from a pipet which dips below the surface of the latter solution. Ruff and Jeroch⁵² have also studied the reaction between nearly neutral sulphite solution and iodine; they state that irregularities are entirely due to the oxidizing effect of the air. They furthermore conclude that no thionates are formed when iodine and sulphide solutions interact.

The necessity of carrying on the titration in acid solution prompted the use of N/50 KOH in the pipet. Sufficient acid was added to a solution of 10 c.c. of N/10 iodine diluted to 100 c.c. to neutralize the total alkali. The absorbing pipet was detached from the manifold, and the contents added slowly beneath the surface of the iodine solution while stirring constantly. The excess of iodine was titrated with N/10 $\text{Na}_2\text{S}_2\text{O}_3$ with a starch indicator. In order to increase the titration values, N/100 iodine was used in subsequent analyses. Only 2 c.c. of N/100 iodine was utilized by the K_2S present, when theoretically 100 c.c. of gas containing 2% H_2S should require 23 c.c. of this concentration of iodine. It is not unlikely that the presence of bicarbonates interfere with the reaction as is the case in the As_2O_3 method. If the gaseous sulphur compounds are converted into K_2S , the instability of this compound in weak solution probably causes its oxidation during the longer period which this dilute alkali requires to absorb completely the CO_2 fraction. Some of these various methods which have been carefully tried are faulty and merely copied from one publication to another without verification by the authors. They are not applicable for the analysis of such small amounts of gas as are available in bacterial metabolism.

(c) *Gravimetric Analysis of Absorbent Containing H_2S .*—Repeated analyses showed in 3 days a total sulphur metabolism of 20-30 mg. This sulphur contained in 100 c.c. of medium when converted into a

⁵⁰ Ztschr. anal. Chemie, 1906, 45, p. 541.

⁵¹ Ztschr. angewicht. Chemie, 1904, 17, p. 557.

⁵² Berl. Deutsch. Chem. Ges., 1905, 38, p. 409.

BaSO₄ precipitate (70 mg.) was of sufficient weight to minimize the unavoidable errors of the process. This sulphur calculated as H₂S would amount to 2%-3% of the total gas evolved. A gravimetric analysis was attempted on the contents of the absorbing pipet containing 200 c.c. of N/50 KOH used for the absorption of a 100 c.c. gas sample. The weight of the precipitated BaSO₄ approximated 10 mg., which would correspond roughly to 1%-2% H₂S fraction. Any gravimetric procedure in which the resulting total precipitate is so small that 1 mg. variations in check analyses represent a 10% error is not trustworthy. In most of the analyses a 2 mg. difference in duplicate determinations represents an error of less than 1% of the 200 mg. BaSO₄ precipitate. The discrepancies between the gravimetric analysis of sulphur metabolism in the medium and the sulphur caught in the pipet was about 1%.

The explanations the writer offers to account for the failure of the methods discussed to demonstrate the 2%-3% H₂S theoretically evolved from the medium according to gravimetric determinations are the following: (1) Adsorption by the P₂O₅ drier of mercaptans and thioethers. This sulphur would not be caught in the gas analysis but would indicate a loss of sulphur in the medium. (2) Some volatile sulphur compounds might be vaporized before the oxidizing reagent used for the sulphur analysis had time to act. (3) The small gravimetric quantity in the sulphur gas, in whatever form it may occur, is in some inert form by the time the absorbing fluid is ready for the titrating reagent which reacts with the sulphide salt.

(f) *Exact Titration of CO₂ in Order to Estimate H₂S as the Alkali Soluble Remainder.*—The Hesse method⁵³ for titrating exactly the CO₂ in gas was modified to suit our apparatus. A solution of oxalic acid, 5.6325 gm. per liter, 1 c.c. of which is equivalent to 1 c.c. of CO₂ at standard conditions, was made. A clear saturated solution of Ba(OH)₂ was diluted until 200 c.c. of this solution will absorb about 80 c.c. of CO₂. This measured 200 c.c. volume of hydroxide was used in a bubbling pipet with the rear reservoir protected from atmospheric CO₂ by a rubber balloon. After total absorption of the gas sample, the contents of the pipet were washed into a beaker, brought to a boil and the excess hydroxide titrated with the oxalic acid. When the solution was hot, the BaCO₃ became granular, and any bicarbonate was totally converted into the carbonate so that neither of these radicals effect the

⁵³ Dennis, L. M.: Gas Analysis, p. 378.

end point when using phenolphthalein as indicator.⁵⁴ The CO_2 formed an immense amount of white BaCO_3 , which made it difficult to read the end point, although it was allowed to settle as much as possible. This method was checked with commercial CO_2 and nitrogen mixtures. The titration values were about 1% higher than the calculated volume of the CO_2 absorbed. In an attempt to reduce this error, the residual Ba(OH)_2 was filled from the carbonate in an atmosphere of nitrogen. The precipitate was washed with CO_2 free water, the BaCO_3 determined by dissolving it in a known volume of N/10 HCl, and the excess acid titrated with alkali using methyl orange as indicator. These results showed little change in the positive 1% error previously found. Consequently, the simpler direct titration method was continued, making allowance for the 1% positive error encountered. The inaccuracy was due to the exposure to the air, the rather clumsy container used in the hydroxide absorption and the difficulty of reading the true end point in the presence of the heavy precipitate. This method promised another angle from which to approximate the percentage of gases other than CO_2 which were caught in the first pipet. This method is usually employed to ascertain small percentages of CO_2 in air or in respiratory gases; it is unquestionably exact, but when applied to mixtures rich in CO_2 , it has the disadvantages already enumerated.

A pipet of known strength Ba(OH)_2 was substituted for the KOH pipet; after absorption, the oxalic acid titration was carried out in boiling solution with phenolphthalein indicator. This titration value for CO_2 was subtracted from the total absorption volume reduced to standard conditions. This volumetric difference was divided by the lesser volume secured by the titration method, which gave the greatest percentage deviation. The average 1% error of the process was subtracted. This gave a close approximation of the amount of extraneous gas absorbed in the alkaline pipet. The percentage deviation did not vary markedly for different organisms such as *B. botulinus*, *B. sporogenes* and *B. histolyticus*, but was lower in the case of the saccharolytic *B. welchii*. It averaged 4% in the rich glucose peptic digest medium as shown in table 1, but was less in the glucose-peptone solution. Reference to table 1 will also show that this approximate error more than covers the probable H_2S evolution as indicated by the sulphur analyses. Work along this line dealing with glucose peptone medium will be given in detail in the second paper of this series. In general it may be

⁵⁴ Sutton, Francis: Volumetric Analysis, p. 42.

stated that the average error in the CO_2 fraction comparing 10 strains grown in 1% glucose 2% peptone medium incubated 3 days was + 4.1%, while in an incubation of 7 days it was + 3.2%. This indicates that the greater part of the odorous gases were formed in the first 3 days of vigorous growth. In a plain 2% peptone medium incubated for a week which produced a smaller total volume of gas, but a much higher percentage of CO_2 than when 1% glucose is added, the error in the CO_2 fraction averaged 3.4%. This percentage is a little higher than

TABLE 1
THREE-DAY INCUBATION ANALYSES IN PEPTIC DIGEST—1% GLUCOSE MEDIUM

Analysis number	1	2	3	4	5*
Organism	B. botulinus	B. sporog.	B. sporog.	B. welchii	B. sporog.
Strain	38	114	114	57	114
Barometric reading (cm.).....	75.1	75.0	75.0	74.8	75.7
Room temperature (C.).....	21	20	20	24	19
Water jacket temperature (C.).....	19	15	18	20	14
Gas pressure in pump (cm.).....	30	28	25	28	20.4
Gas volume at standard (cc.).....	670	620	590	720	352
Change in Pa.....	7.4-5.8	7.4-6.4	7.4-6.4	7.4-5.0	7.4-6.8
Grams of BaSO_4 lost per 100 cc. medium	0.0548	0.0468	0.0501	0.0018	0.048
Total gm. of sulphur lost.....	0.0377	0.0322	0.0345	(negligible)	0.0286
Theoretical percentage of H_2S evolved	3.94	3.64	4.0	(negligible)	5.7
Percentage foreign gas in CO_2 frac- tion determined by Ba(OH)_2 method	4.6	4.1	4.3	2.0	5.9
Percentage of oxides of nitrogen....	0.2	0.2	0.2	0.4	0.2
Gas Analysis:					
Percentage CO_2	85.0	86.4	86.3	40.1	76.0
Percentage H_2	10.0	10.1	10.1	42.2	23.2
Percentage N_2	4.75	3.5	3.4	17.5	0.8
Total percentage	99.75	100.0	99.8	99.8	100.0
CO_2 : H_2 ratio.....	8.5	8.6	8.6	0.954	3.28

* No glucose added to medium in analysis No. 5.

that obtained for a week's incubation on glucose peptone medium. However, it indicates a different manner of metabolism of the peptone in the absence of glucose, but, on the other hand, the deviation is not sufficiently marked to deserve additional inquiry.

Table 1 is a representative example of the data obtained by some of the methods discussed. Attention is called to the following details in the data; (1) the accuracy with which two separate analyses on flasks of *B. sporogenes* compare in detail (exper. 2 and 3); (2) the negligible amount of sulphur metabolized by *B. welchii*. This result checks well with the small probable error in the CO_2 fraction, which is calculated

approximately by using the $\text{Ba}(\text{OH})_2$ titration method; (3) differences in the sulphur metabolized, the total gas production, and the CO_2/H_2 ratio of *B. sporogenes* (exper. 3 and 5) when glucose is omitted from the rich peptic digest medium; (4) the close correspondence between the estimated percentage of H_2S and the foreign gas estimated by the $\text{Ba}(\text{OH})_2$ titration for CO_2 ; in fact, the latter method, although only approximate, gives results slightly higher in percentages than any extraneous gas demonstrated.

The analytical methods customarily applied to ascertain the nature of an unknown gas constituent were not applicable. The Dumá bulb used for estimating molecular weights calls for an exact barometer, pressure gage, measuring system, and drying apparatus if the volume of gas weighed is to be resolved by percentages into its component factors. This expensive type of apparatus was not available. A large volume of gas is necessary for spectroscopic electrical discharge analysis in an interferometer, and then but one unknown gas can be estimated and not traces of several gases. The use of colorimetric buffer methods in which readings of color changes are taken at intervals has been used in bacterial respiration work when only minute traces of CO_2 are produced. Fractionation of the gases by freezing the contents of a Dewar flask by means of liquid air would not be likely to separate the CO_2 and H_2S , as there are only a few degrees between their respective liquefaction temperatures. Nothing is known of their relative solubilities in one another in the liquid state at low temperature. The measurement of the electrical conductivity of a fresh $\text{Ba}(\text{OH})_2$ absorbent and the lessened conductivity after absorption due to the presence of BaCO_3 seems the most promising of any of these physical chemical methods. The electrical conductivity measurements necessitate the use of a wheatstone bridge, phones and inch square platinum electrodes. The physical tables give no data on the conductivity of the soluble BaS or BaSH present. These would have to be computed experimentally. The sulphides of barium, which are highly soluble, are titrated along with the soluble hydroxide in the Hesse method. They would not complicate the Hesse reaction as much as the conductivity measurements.

After trying to adapt a number of common chemical procedures to the problem under consideration, it was concluded that the Hesse $\text{Ba}(\text{OH})_2$ method gave the greatest degree of satisfaction under the existing circumstances. The data obtained are accurate within a pos-

sible error of 0.5%. The results correlate well with gravimetric sulphur analyses; consequently no more complicated means of solving the problem of the odorous gases produced by a number of anaerobic organisms have been investigated. Although the use of conductivity measurements on the $\text{Ba}(\text{OH})_2$ absorbent seems likely to yield more accurate results, this was not undertaken on account of the complicated equipment necessary. In case a synthetic medium is available for these organisms instead of a complex peptone, more elaborate methods will be used to supplement the definite chemical analyses.

After the investigation of the H_2S methods had been completed, Heath and Lee⁵⁵ published a report, which considers the errors in the determination of H_2S in water. They have encountered, although to a lesser degree, the same difficulties as related in the present report. These authors recommend that "the use of the iodimetric process for the determination of H_2S in natural waters be discontinued. In order to eliminate errors due to the action of nitrites, nitrates, and alkali salts, we suggest that natural waters be tested for H_2S by the colorimetric method of W. Mecklenburg and F. Rosenkränzer,⁵⁶ in which methylene blue is formed. The need of a qualitative as well as a quantitative method is further shown by the fact that some of the samples from Devil's Lake gave no test by this latter method, but positive results were obtained by the iodimetric method."

Colorimetric determinations with this methylene blue reaction have been made on the N/50 KOH absorber. An excellent deep blue color was obtained from the absorbed 100 c.c. gas sample. Approximately 2% H_2S was calculated for the gas evolved during 3-day incubation on a 1% glucose-2% peptone medium. However, no analyses for the total sulphur metabolized have as yet been undertaken. Salts such as NaCl , CaCl_2 and urea have no effect on the accuracy of this color reaction. So far as is known, traces of mercaptans and thioethers may react colorimetrically in the same manner as H_2S . Further studies with this method are in progress in order to determine the degree of accuracy with which this method can be adapted to the determination of H_2S in metabolic bacterial gases.

SUMMARY

A brief discussion of the literature dealing with gas analytical methods applied to biologic problems is presented.

The Toepler high vacuum mercury pump with modifications and connections which make it efficient for drawing off accurately gas samples from bacterial cultures is described. The Burrell gas analysis apparatus, which gives results correct to 0.1% can be well adapted and furnishes satisfactory analytical results. A modified Kjeldahl flask is employed as a receptacle for the cultures. A standardized technic is described.

⁵⁵ Jour. Am. Chem. Soc., 1923, 45, p. 1643.

⁵⁶ Ztschr. f. inorg. Chem., 1914, 86, p. 143.

The main gases produced by anaerobic bacteria are CO_2 , H_2 and N_2 . However, the odor in many cases suggests the presence of organic amines and sulphur compounds of a volatile nature. The presence of H_2S has been proved. A number of authenticated methods for the quantitative estimation of the H_2S has been tried, but the results failed to check accurately the sulphur analysis made on the culture medium. These studies are described in detail. It is shown that the unknown fraction of the gas, which is present as a small percentage, can be estimated by the exact determination of the CO_2 in the alkali-soluble fraction by absorbing it in $\text{N}/10 \text{ Ba}(\text{OH})_2$ according to the method of Hesse. The main part of the experimental data will be given in the second paper dealing more fully with the correlation of the results and their metabolic significance.

GASEOUS METABOLISM OF SOME ANAEROBIC BACTERIA

XX. EXPERIMENTAL DATA

BELLE G. ANDERSON

From the George Williams Hooper Foundation for Medical Research, University of California Medical School, San Francisco

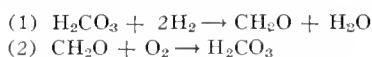
Aided by grants from the National Cannery Association, the Cannery League of California and the California Olive Assn.

INTRODUCTION

In the preceding paper, the methods best adapted for collecting information concerning the gaseous metabolism of anaerobic bacteria, have been selected. Preliminary experiments to determine the nature of the gases and the technic employed have been described in detail. The data reported in subsequent pages furnish information regarding the physiology of anaerobic metabolism. A detailed description of the experiments is justified on account of the scarcity of published work dealing with the same subject.

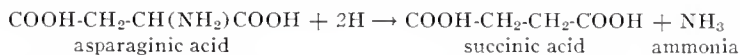
The studies were primarily undertaken to secure data which might be of assistance in the interpretation of anaerobic spoilage in canned goods, especially the type due to *B. botulinus*. It was furthermore expected that the results might prove of diagnostic value. Secondly, additional data were desirable in order to supplement other biochemical studies on anaerobic fermentation conducted in this laboratory. The procedures followed in gas metabolism studies must, of course, be standardized and capable of duplication in order that the data can be used for comparisons in the future. For this reason beef heart or veal infusion broths as a basic medium were considered undesirable. "Difco" peptone in varying percentages, taken from a 6 pound pooled sample, was used as a base. The composition of the gas evolved from this medium was determined. The culture fluid has been varied by the addition of inorganic salts, different carbohydrates, and combinations of these 2 substances. The effect of different periods of incubation was also investigated. In one instance, the volume of medium was reduced from the usual 300 c.c. to 150 c.c. in order to study the effect of the increased vacuum space on the volume of the gas, or its components. As many as 25 strains of anaerobes have been grown in the standard medium.

It must be remembered that the gas samples analyzed represent only one of the end products of the fermentation at the selected time the flask is removed from the incubator. The nascent gases are likely to enter into other reactions in the medium, and may in themselves be a source of energy for the metabolic processes. The latter statement is demonstrated by the activity of certain photosynthetic organisms, which synthesize their food from simple elements. To W. Ruhland,¹ who in a recent address discussed autotrophic (photosynthetic) and heterotrophic bacteria, is attributed the following statement: "It is definitely established that the chemical energy of free hydrogen is utilized by bacteria for H_2CO_3 assimilation." He mentions different theories dealing with the manner of reaction. Ruhland disagrees with the conceptions of both Kaserer and Niklewski, but believes in a direct slow combustion of the hydrogen. According to Kaserer, the process passes through two stages:



It is plain that the ratio of the volumes of the two gases used up, i. e., H_2/O_2 , equals two. However, Ruhland describes an experiment in which the volumes of gas utilized may be ascertained. In his experiment, the ratio of hydrogen to oxygen used by photosynthetic bacteria ranged between 3 and 4, indicating the use of little oxygen. He considers the process a direct consumption of the hydrogen without any secondary synthesis into carbonic acid. This interpretation may be correct under the experimental conditions chosen by Ruhland and may have some bearing on the problem under consideration. Unfortunately, the course of such a reaction could only be studied in a synthetic medium and not in a complex peptone solution.

Besides the probable reaction of the nascent gases among themselves, the hydrogen may be consumed by some unsaturated chemical group, or unstable radical formed during the decomposition of the medium. Harden² found that hydrogen produced by *B. coli communis* in fermented glucose or mannitol reduced the amino group of sodium asparaginate, which served as the sole source of nitrogen. The asparaginic acid is reduced to ammonium succinate. The amount of the latter can be estimated by determining the amount of ammonia in the culture. By calculating the ammonium succinate produced, the hydrogen used in the reduction may be ascertained. The total hydrogen concerned in this process corresponds well to the amount obtained from glucose and mannitol decomposition using other sources of stable nitrogenous substances. The reaction of deamination is explained by the following equation:



Numerous chemical determinations prompted Harden to represent the breakdown of glucose in the following molecular equation:

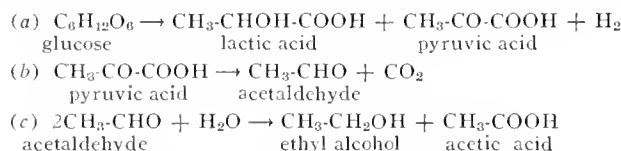


However, no certainty exists, even if this reaction occurs, that equal volumes of CO_2 and H_2 can be recovered, as these gases may in turn play a part in other reactions. Harden states that the equation "somewhat overestimates the amount of carbon dioxide and hydrogen which are actually found," but the gas ratio does not depart widely from unity.

¹ Berl. Deutsch. Botan. Ges., 1922, 40, p. 180; Chem. Abstr., 1922, 16, p. 3921.

² Jour. Chem. Soc. Trans., 1901, 79, p. 623.

Neuberg and his associates nearly 20 years later, verify quantitatively the end products of Harden's glucose fermentation equation. However, they have demonstrated certain intermediary products which elucidate the chemical change. Neuberg and Nord³ trace the decomposition of glucose by *B. coli* through the following 3 stages before the same end products are reached:



The manner of the primary reduction from 6 to 3 carbon atoms is not certain. However, Neuberg considers methylglyoxal (pyruvic aldehyde—CH₃—CO—CHO) as the first 3 carbon atom compound formed. It is obtained by the migration of H- and OH-ions during the decomposition of one molecule of glucose necessitating the loss of two molecules of water. The substance is identified by precipitation of the osazone with phenylhydrazine. The ketonic aldehyde can be chemically converted into both acids represented in equation (a); into lactic acid by the addition of a molecule of water, and into pyruvic acid by oxidation. This lowest member of the α-ketonic acids can be regarded either as an oxidation product of methylglyoxal or as a dehydration product of glyceric acid (CH₂OH—CHOH—COOH). There is definite evidence of the formation of both glycerol and pyruvic acid from the enolic form of methylglyoxal CH₂:C(OH)—CHO. Aside from the steps in the formation of either the ketonic acid (a) or the acetaldehyde (b), it is interesting to note that both compounds have a "carbon double bond oxygen linkage" capable of reducing nascent hydrogen. The reduction of pyruvic acid would yield lactic acid; acetaldehyde would be converted into ethyl alcohol and the percentage of gaseous hydrogen be diminished.

According to the data of Neuberg, Rainfurth and Sandberg,⁴ α-ketonic acids and their derived aldehydes, compounds of the purine group and their derivatives, or substances containing purine nuclei, markedly accelerate the fermentation of sugar. The invigorating effect of compounds of this nature can be explained by their chemical function as "H-acceptors." The general theory of stimulation advanced by these authors is that: "substances capable of acting as hydrogen acceptors have an accelerating action upon the process of fermentation." The hydrogen evolved by proteolytic anaerobes is doubtless absorbed by such reducing compounds or by deaminization reactions, and consequently a low percentage remains in the gas sample. The present day knowledge on the fermentation processes is well summarized in the monograph of Pringsheim⁵ and of Fuchs,⁶ and does not need repetition.

These points are mentioned to emphasize the fact that the analyzed gas samples taken at an arbitrary time are themselves only end products, left after certain reactions have already occurred in the complex peptone medium. Nevertheless, these analyses may prove reliable means to determine whether a fermentation in a given medium is entirely saccharolytic, proteolytic, or a combination of the two processes in a given period of time.

³ Biochem. Ztschr., 1919, 96, p. 133.

⁴ Biochem. Ztschr., 1921, 121, p. 215; Physiol. Abstr., 1922, 6, p. 534.

⁵ Abderhalden, E.: Handbuch der biochemischen Arbeitsmethoden, 1922, 12, pp. 41-105.

⁶ Sammlung chemischer und chemisch-technischer Vorträge, 1922, 27, p. 1.

PERSONAL INVESTIGATIONS

A copy of a single analysis, which shows the form found most convenient for daily records, is presented in table 1. A sample of gas, if its volume permits, is always analyzed twice, and the percentage results averaged. Whenever sufficient gas is available, a 100 c.c. sample is taken for each analysis in order to reduce the errors, unavoidable with this kind of analysis apparatus. The analysis of this quantity of gas yields results which are accurate to within 0.1%. The changes in H-ion concentration are determined colorimetrically by using the buffers and the indicators recommended by Clark.⁷ It must be remembered that the

TABLE 1
DAILY ANALYSIS RECORD

	Cc.	%	Average		Cc.	
			%	%		
Sample.....	75.6	97.4	B. sporogenes 114
After KOH absorption.....	14.8	97.4	Planted May 19, a. m.
Absorbed CO ₂	60.8	80.5	80.6	80.8	18.7	Grew May 20 a. m.
After phosphorus absorption..	14.8	18.7	Opened May 22 a. m.
Absorbed O ₂	0.0	78.7	Change P _H = 7.4-6.3
Intake of air.....	76.1	18.7	Room temperature 20 C.
Total volume combusted.....	90.9	0.0	Barometer 750 mm.
O ₂ content of air.....	16.0	61.6	Total volume gas = 180 cc.
N ₂ content of air.....	60.1	80.3	Standard volume gas = 165 cc.
After combustion.....	73.0	12.9	
Contraction.....	17.9	48.7	
After phosphorous absorption.	63.0	57.5	Medium
Unused O ₂	10.0	22.8	Peptone 2%
Combusted O ₂	6.0	52.2	Salicin 1%
Combusted H ₂	11.9	15.7	15.6	15.6	5.3	
Residual N ₂	2.9	3.8	3.7	3.6	7.6	
Total percentage.....					15.2	
					3.5	
					99.9	

final reaction is more alkaline than the P_H, which is found in ordinary cultures saturated by their gases. The medium is free from CO₂, which is the most soluble gas and an anhydride of a weak organic acid; its loss decreases the acidity. Since most of the mediums have a final P_H below 7 after complete exhaustion, ammonia is the only gas retained in the form of its acid salts in the solution.

The total amount of gas was collected in the eudiometer H, which is part of the Topley pump system shown in fig. 1 of the preceding paper. This eudiometer measures the volumes accurately within 2 c.c., when the evacuation is complete, as indicated by the manometer registering the constant pressure of the water vapor in the system. This volume is reduced to 760 mm. and 0 C., and is reported at standard conditions in all the tables of this paper. The fraction soluble in KOH is designated as CO₂ in order to avoid confusion. However, it has been shown by preliminary experiments that gases other than CO₂ may be absorbed. The KOH in the pipet has a rank odor after absorption, due to the retention of other acid gases such as H₂S, thioethers and volatile

⁷ Determination of Hydrogen Ions, 1922.

amines. These amines resemble ammonia in their alkaline reaction, and are extremely soluble in any aqueous solution. The presence of 0.1-0.2 c.c. of oxygen in a sample indicates a slight air leakage in the connections during the transfer of the gas from the eudiometer to the analysis buret. Corresponding correction can be made in the analysis for this small amount of air leakage. If larger amounts of oxygen occur, the sample must be discarded. A small crack in the flask itself may alter the anaerobic conditions, and consequently influence the nature of the gas evolution.

TABLE 2
DUPLICATION OF ANALYTICAL RESULTS

Organism	Ex- peri- ment	Strain	Additions to 2% Peptone Medium	Appear- ance of Medium	P _H Change	Gas Volume at Stan- dard, Cc.	CO ₂ %	H ⁺ %	N ₂ %	Sum %	Ratio CO ₂ H ₂	Time Inter- val, Days
B. botulinus	1	38	1% glucose	Black	7.4-6.6	177	87.0	7.55	5.3	99.85	11.5	59
		38	1% glucose	Black	7.4-6.5	205	86.0	7.2	7.5	99.7	11.9	
	2	40	1% glucose	Black	7.6-6.6	302	97.5	0.9	1.4	99.1	107.0	47
		40	1% glucose	Black	7.4-6.4	270	96.8	0.8	2.3	99.9	120.0	
	3	19	1% salicin	Black	7.4-6.4	325	72.6	25.2	2.1	99.9	2.88	163
		19	1% salicin	Black	7.4-6.2	425	73.0	24.6	2.28	99.88	2.96	
B. sporogenes	4	114	1% salicin	Black	7.4-6.3	165	80.5	15.7	3.8	100.0	5.1	191
		114	1% salicin	Black	7.6-6.2	250	80.5	16.8	2.6	99.9	4.5	
		114	1% salicin	Black	7.4-6.4	180	81.9	17.1	1.9	99.9	4.7	
	5	48	1% salicin	Black	7.4-6.4	265	89.1	5.7	4.9	99.7	15.6	158
		48	1% salicin	Black	7.4-6.6	215	88.5	5.45	5.7	99.8	16.2	
B. welchii	6	10/46	1% salicin	Yellow	7.4-5.6	325	48.8	49.8	1.3	99.9	0.98	115
		10/46	1% salicin	brown Yellow brown	7.4-5.4	310	48.0	49.0	2.6	99.6	0.97	
	7	10/46	1% glucose + 1% NaNO ₃	Very yellow	7.4-6.3	250	89.5	1.48	8.9	99.88	60.0	146
		10/46	1% glucose + 1% NaNO ₃	Very yellow	7.4-6.0	285	90.9	2.0	7.0	99.9	45.4	
	8	57	1% glucose + 1% NaNO ₃	Very yellow	7.4-6.6	182	88.3	2.95	8.7	99.9	30.0	169
		57	1% glucose + 1% NaNO ₃	Very yellow	7.4-6.4	200	91.0	2.1	6.0	99.1	43.3	
	9	57	1% salicin	Brown- ish	7.4-7.1	30	24.9	67.0	7.7	99.6	0.37	164
		57	1% salicin	Brown- ish	7.4-7.2	21	18.8	67.1	12.7	99.6	0.28	

A 3 day incubation period at 37 C. was employed in all cases given here.

For a long time a pipet of acid CuCl has been used to detect the presence of CO. It was later discarded; even while dealing with a large number of samples, no significant contraction has been obtained. No evidence of nitric oxide, carbon monoxide, methane, or other hydrocarbon gases has been obtained by absorbing in a 10% KOH pipet after combustion. In several sample analyses published in the literature, the hydrogen is simply computed as two-thirds of the observed contraction. It is advisable, however, to check the combustion contraction value, as shown in table 2, by absorbing the unused oxygen. The

oxygen, which has been consumed in burning the hydrogen should be exactly half of the volume of the hydrogen calculated from the contraction reading. This additional measurement of the volume absorbed by the phosphorus after combustion serves as a check for the residual nitrogen present. Any leakage occurring during combustion is plainly recognized by taking this last reading. Carbon dioxide free air is used for combustion in preference to pure oxygen. The combustion is not so violent, lessening breakage, and avoiding the burning of the residual nitrogen to form oxides. The total percentage accounted for is an index of the accuracy of the analysis as a whole.

DUPLICATION OF ANALYTICAL RESULTS

Table 2 represents a series of duplicate determinations with the same strain and medium. The time interval between the check analyses varied from 47 to 191 days, or roughly from 1 to 5 months. During this time, the stock cultures were kept in beef heart medium. The gas flasks were filled with different lots of basic 2% peptone medium. Primarily most of those analyses were duplicated in which the results from the first calculation seemed irregular compared with data already obtained from other strains of the same organism. Furthermore, they constitute substantial evidence of the constancy of the process and the nature of the gas metabolism. The volumes of gas recovered from duplicate flasks varied considerably, i. e., from 9 c. c. to 100 c. c. (exper. 9 and 3, respectively). Regardless of this fact, the nature of the gas from identical strains did not change appreciably, as can be seen by a comparison of the CO_2/H_2 ratios of each set of duplicates. In comparing the differences which occur in this ratio a few points with regard to the character of these figures deserve consideration. When the CO_2/H_2 ratio is 1 or less than 1, as in exper. 6, the deviation is 0.01, which represents an error of less than 1%. In contrast to this small percentage deviation is the large numerical difference of 14.6 in exper. 7, which is a 28% deviation from the mean value of the CO_2/H_2 ratio, equal to 52.7 numerically. However, in this particular experiment the variation in percentages of CO_2 and H_2 are 1.4% and 0.5%, respectively. Such a large ratio results when the percentage of hydrogen is very low. The actual error is no more alarming than in exper. 6. However, the relative proportion between this high CO_2 value and the exceedingly low H_2 value, as shown in the duplicate CO_2/H_2 ratios, may be misleading. In the ratios (exper. 1 and 4) ranging in value from 5 to 10, the deviations are numerically 0.4 and 0.6, respectively. This makes the percentage deviation from the mean value for exper. 1 equal to 3.4% and for exper. 4 equal to 12.5%. But when looking at the percentages of the component gas fractions, the variations are actually only

about 1%. The variation in percentages of different gases between duplicate determinations is about 1% for CO_2 and less than 1% for H_2 differences. When the percentage of H_2 is small compared to the CO_2 fraction, making the CO_2/H_2 ratio a large number, the latter magnifies any slight alterations in the percentage figures used to calculate this ratio.

It is evident that the reactions are practically constant, provided the figures as given in table 2 are interpreted in the light of the preceding discussion. The apparent error is large, when the gas volume is scant, as seen in exper. 9; for example, the two numerical ratio values differ by 0.09, but this small figure nevertheless gives about 28% deviation from the mean value. A single analysis of such small gas volumes (25 to 30 c.c.) in a 100 c.c. buret has not an accuracy of 0.1%, which is obtained from larger samples (150 c.c. or more). Contrasted to this 28% deviation are those of exper. 3 and 6, 1% and 0.3%, respectively, in which the volumes are larger, although they may not check within 100 c.c. However, in exper. 3, the larger volume (300-400 c.c.) of an active metabolic process show CO_2/H_2 ratios, which check within 2.7% from their mean value of 2.92 for the two determinations made 5 months apart. In view of these unavoidable variations in the total volumes and the large figures indicated in the CO_2/H_2 ratios, the comparisons in the data must be taken as generalizations. No undue significance should be attributed to small, numerical differences in the analytical data secured in separate experiments, studies on various species of bacteria and separate strains of the same organisms. With the exception of *B. welchii*, the variations among the strains grown in different mediums are far more striking than those characteristic for the species propagated in the same environment. However, the tendency of the strain variations are constant over a long period of observation, and the values can be checked to such a degree of accuracy that confusion with other strains of the same species is impossible.

GAS METABOLISM STUDIES

The records presented in the tables are based on gases evolved from culture seeded in 300 c.c. of basic 2% Difco peptone medium, modified in different experiments by the addition of known strength sterile glucose, salicin, sodium chloride, etc. Culture in flasks (modified Kjeldahls) have been incubated at 37 C. for 3 or seven day periods, according to the type of medium employed. The time of planting and the subsequent growth have been controlled and kept as uniform as

possible. The care of stock cultures, production of standard inoculums, and precautions against contamination are fully detailed in the first paper dealing with the technical aspect of the problem.

Plain Peptone Fermentation.—In table 3A are recorded the complete data on the fermentation of 2% peptone solution (7 days' incubation) by the growth of 24 strains of bacteria. The volume of gas evolved by proteolytic organisms never exceed 100 c.c., while a saccharolytic organism, such as *B. welchii*, generated approximately 25 c.c. The proteolytic organisms form mainly CO_2 and give CO_2/H_2 ratios varying from 6 to 45 in numerical value. *B. welchii* produces little gas, but the composition is much higher in H_2 , giving CO_2/H_2 ratios varying from 0.15 to 0.4. *B. bifermentans*, which in many respects resembles *B. welchii*, is capable of handling the peptone in a decidedly proteolytic manner. It produces a considerable volume of gas and a CO_2/H_2 ratio of 45.1. Two strains of vibriion septique give ratios approximating 1 and resemble *B. welchii* more closely than *B. bifermentans*.

The results previously obtained with the use of the Hesse method for the direct CO_2 determination are included in this table. The CO_2 was estimated by the volume absorbed in $\text{Ba}(\text{OH})_2$ and also by titrating the excess of alkali left after absorption. The difference between these two volumes determined is divided by the lesser titration volume and gives the greatest positive percentage deviation of this fraction. This figure represents the probable percentage of the alkali soluble fraction, which is not pure CO_2 but a mixture of CO_2 , H_2S , volatile organic sulphur compounds and amines of low boiling point. The error in the CO_2 fraction averages 3.4% when dealing with proteolytic organisms, such as *B. botulinus* and *B. sporogenes*. The individual variations are high with strains which give high CO_2/H_2 ratios. These figures are larger than the estimated percentage of H_2S formed in peptic digest medium, table 1 of the previous paper. The buffer substances in the peptone prevent an acid P_H in the medium. This neutrality may lead to a small amount of CO_2 retention in the fluid in form of carbonates or bicarbonates. These substances have not been determined.

It is interesting to note that a highly proteolytic organism such as *B. histolyticus* forms no H_2 from peptone. In fact, only one instance is recorded in which this organism produced H_2 (table 8A 0.5% peptone medium + 1% glucose). According to the data in table 2, it is evident that the greatest degree of constancy in the CO_2/H_2 ratio is typical for representatives of the *B. welchii* group. The black appearance of most

TABLE 3

(A.) FERMENTATION OF DIFCO PEPTONE (2%) AFTER A 7-DAY INCUBATION PERIOD

Organism	Strain	Appearance of Medium	pH Change	Volume at Standard, Cc.	CO ₂ %	H ₂ %	N ₂ %	Sum, %	Ratio CO ₂ / H ₂	Error in CO ₂ Fraction, %
1. B. botulinus.....	97	Black	7.4-7.2	79	82.0	13.6	4.1	99.7	6.0	+2.9
2. B. botulinus.....	19	Black	7.4-7.2	63	85.5	7.5	6.8	99.8	11.4	+3.2
3. B. botulinus.....	62	Black	7.4-7.1	120	93.5	3.6	2.7	99.7	25.9	+3.5
4. B. botulinus.....	34	Black	7.4-7.1	125	91.5	3.3	5.0	99.8	27.7
5. B. botulinus.....	53	Black	7.4-7.3	46	81.0	15.6	3.2	99.8	5.2	+2.6
6. B. botulinus.....	38	Black	7.4-7.2	120	92.5	3.0	4.1	99.6	30.8	+4.1
7. B. botulinus.....	26	Black	7.4-7.1	130	89.5	4.0	6.3	99.8	21.4
8. B. botulinus.....	40	Black	7.4-7.2	47	92.0	1.0	7.1	100.0	92.0	+4.2
9. B. sporogenes.....	46	Black	7.4-7.0	118	89.5	3.5	6.0	99.0	25.6	+3.6
10. B. sporogenes.....	114	Black	7.4-7.2	163	91.6	5.0	3.3	99.9	18.3	+2.6
11. B. sporogenes.....	Y	Black	7.4-7.2	118	92.5	1.3	5.9	99.7	70.1
12. B. sporogenes.....	48	Black	7.4-7.0	164	90.7	2.7	5.7	99.1	33.6	+4.0
13. B. welchii.....	57	Brown	7.4-7.1	42	25.9	64.5	9.5	99.9	0.40	Av. +3.4
14. B. welchii.....	10/46	Brown	7.4-7.2	29	20.8	70.5	8.7	99.9	0.30	
15. B. welchii.....	26	Brown	7.4-7.2	16	12.5	83.0	4.6	100.1	0.15	
16. B. welchii.....	2	Brown	7.4-7.2	17	35.8	46.3	16.8	99.9	0.77	
17. B. welchii.....	129	Black	7.4-7.2	19	19.0	58.5	22.3	100.0	0.33	
18. B. welchii.....	36	Brown	7.4-7.2	28	33.4	57.7	8.8	99.9	0.58	
19. B. welchii.....	135	Brown	7.4-7.3	30	18.3	66.7	14.3	99.3	0.27	
20. Vibrion septique.....	RNS	Black	7.4-7.4	18	45.2	43.2	11.2	100.0	1.04	
21. Vibrion septique.....	99-139	Black	7.4-7.2	28	40.0	43.0	17.0	100.0	0.93	
22. B. centrosporogenes.....	82	Black	7.6-6.8	50	82.5	2.1	15.3	99.9	37.4	
23. B. histolyticus.....	WV	Black	7.4-6.6	70	92.5	0.0	7.0	99.5	
24. B. bif fermentans.....	62	Black	7.4-7.2	46	90.0	2.3	7.5	99.8	45.1	

(B) EFFECT OF HCOONa (1%) ON FERMENTATION OF PEPTONE (2%) AFTER 7 DAYS' INCUBATION

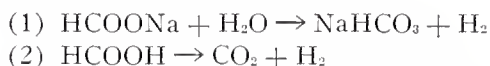
1. B. botulinus.....	97	Brown	7.4-7.2	78	74.5	20.8	4.7	100.0	3.58	
2. B. botulinus.....	19	Brown	7.4-7.2	100	53.0	37.8	9.3	100.1	1.4	
3. B. botulinus.....	62	Brown	7.4-7.1	100	80.0	14.7	5.0	99.7	5.4	
4. B. botulinus.....	34	Brown	7.4-7.2	117	90.3	5.3	4.4	100.0	17.0	
5. B. botulinus.....	53	Brown	7.4-7.2	35	53.8	39.8	6.2	99.8	1.35	
6. B. botulinus.....	39	Brown	7.4-7.1	70	77.8	18.5	3.7	100.0	4.2	
7. B. botulinus.....	38	Brown	7.4-6.8	125	82.7	13.4	3.8	99.9	6.17	
8. B. botulinus.....	26	Brown	7.4-7.1	135	86.5	11.4	2.0	99.9	7.6	
9. B. sporogenes.....	46	Black	7.4-7.0	125	77.1	15.7	7.0	99.8	4.9	
10. B. sporogenes.....	114	Black	7.4-7.2	175	83.6	14.2	2.1	99.9	5.9	
11. B. sporogenes.....	Y	Black	7.4-7.0	118	86.8	10.2	2.8	99.8	8.5	
12. B. sporogenes.....	48	Black	7.4-6.9	125	75.4	17.8	6.6	99.8	4.2	
13. B. welchii.....	57	Brown	7.4-7.2	25	18.5	69.9	10.9	99.3	0.26	
14. B. welchii.....	10/46	Brown	7.4-7.4	18	12.8	79.2	8.0	100.0	0.16	
15. B. welchii.....	26	Brown	7.4-7.2	22	7.3	90.8	2.0	100.1	0.08	
16. B. welchii.....	2	Black	7.4-7.2	19	13.2	80.0	6.8	100.0	0.17	
17. B. welchii.....	129	Brown	7.4-7.2	17	11.2	77.0	11.8	100.0	0.15	
18. B. welchii.....	36	Brown	7.4-7.2	30	14.6	75.5	10.0	100.1	0.19	
19. B. welchii.....	135	Brown	7.4-7.2	35	19.9	66.3	13.4	99.6	0.30	
20. B. centrosporogenes.....	82	Black	7.4-6.9	80	94.3	3.7	2.0	100.0	25.5	
21. B. bif fermentans.....	62	Brown	7.4-7.2	40	81.3	9.4	9.0	99.7	8.7	

(C.) EFFECT OF NaCl (1%) ON FERMENTATION OF PEPTONE (2%) AFTER 7 DAYS' INCUBATION

1. B. botulinus.....	97	Brown	7.4-7.2	90	78.2	17.8	4.0	100.0	4.4	
2. B. botulinus.....	62	Black	7.4-7.2	107	83.0	13.7	3.2	99.9	6.1	
3. B. botulinus.....	38	Brown	7.4-7.3	80	82.9	10.8	6.1	99.8	7.7	
4. B. sporogenes.....	46	Black	7.4-7.0	130	80.1	11.3	8.4	99.8	7.1	
5. B. sporogenes.....	Y	Black	7.4-7.0	125	90.3	3.0	6.5	99.8	30.1	
6. B. sporogenes.....	48	Black	7.4-7.0	153	79.8	15.1	5.0	99.9	5.3	
7. B. welchii.....	26	Brown	7.4-7.2	15.4	10.4	88.5	1.0	99.9	0.12	
8. B. welchii.....	2	Brown	7.4-7.2	17.3	15.0	66.5	18.5	100.0	0.23	
9. B. welchii.....	129	Brown	7.4-7.2	15.0	14.0	60.5	25.3	99.8	0.23	
10. B. welchii.....	36	Brown	7.4-7.3	35.0	16.3	72.2	11.1	99.6	0.23	
11. B. welchii.....	135	Brown	7.4-7.4	20.0	17.4	80.7	1.9	100.0	0.22	
12. Vibrion septique.....	99-139	Black	7.4-7.2	11.0	39.2	56.8	3.9	99.9	0.69	
13. B. centrosporogenes.....	82	Black	7.4-7.0	63.0	89.3	3.5	7.0	99.8	25.5	
14. B. bif fermentans.....	62	Brown	7.4-7.2	35.0	87.0	7.6	5.3	99.9	11.4	

of the mediums indicates active H_2S evolution and formation of FeS . The percentages of nitrogen are much higher than in other mediums containing carbohydrate, which indicates an initial denitrification process, especially noticed with strains of *B. welchii* and *Vibrio septique*. The percentage figures magnify the number of cubic centimeters of nitrogen evolved during a week's time, as the total volume produced by the two bacteria is only 17 c c. to 30 c c.

Effect of Sodium Formate and Sodium Chloride on Peptone Fermentation.—Having secured data on the amount and the nature of the gases evolved by anaerobic bacteria in a peptone solution, the effect of carbohydrates on the fermentation has been investigated. The formation of certain organic acids precedes the evolution of gaseous products from the carbohydrate, as indicated by the glucose decomposition equation discussed in the introduction. Formic acid is the simplest of all organic acids, and it is considered by some workers the last decomposition product before the gas is formed. In order to elucidate the complex process of carbohydrate decomposition, some means had to be chosen to study the breakdown of some related compound possessing a simpler chemical structure. Pakes and Jollyman⁸ reported on the bacterial decomposition of formic acid into CO_2 and H_2 , using sodium formate as the inorganic salt of formic acid. They conclude that as a rule organisms which ferment d-glucose will likewise decompose sodium formate and formic acid according to the equations:



Reliable apparatus was not used for extracting the gases for analysis, and much of their work was done on a basic meat-broth medium incubated for 3 or 4 weeks. They realized that meat extract and peptone were open to criticism; they employed 2% peptone water, 0.5% sodium chloride and varying percentages of sodium formate. Regarding the use of 2% peptone they made the following statement: "This peptone solution, when inoculated with the various bacteria mentioned above, yields no trace of gas." The addition of sodium formate and sodium chloride to the peptone lead them to conclude that the gas evolved comes from the formate. No doubt the formate changes the reaction, but *B. coli*, *B. prodigiosus*, etc., yield gas from plain peptone when proper means for the recovery of the gas are used. Pakes and

⁸ Jour. Chem. Soc. Trans., 1901, 79, p. 386.

Jollyman use the percentage of hydrogen formed as an index of the amount of formate decomposed. The anaerobic organisms employed in this study ferment glucose actively. It was expected that the kinetics of the carbohydrate fermentation could be more readily understood by analysis of the effect produced by the addition of 1% sodium formate to a peptone solution. It was considered advisable to omit the 0.5% sodium chloride used by Pakes and Jollyman. A separate series of cultures in a peptone solution with 1% sodium chloride has been studied. In table 3, B and C, the data of these experiments with the two salts having a common metallic ion are reported. The addition of these salts did not markedly alter the P_H changes from those previously noted in plain 2% peptone. However, sodium formate as well as sodium chloride influence the gas metabolism of the medium, as is well shown by a change in the CO_2/H_2 ratios. The medium does not turn as black as in the case of plain peptone fermentation, which indicates that less H_2S is evolved.

Discussion of Comparative Peptone Fermentations.—In Table 4 the data are summarized which lend themselves to a comparison of the behavior of identical bacterial strains grown in the peptone mediums described in the preceding pages. The volumes indicate roughly the activity of the fermentation, and the CO_2/H_2 ratios show the changes in the constituent gases. The experimental conditions remained identical with reference to temperature, time of incubation and volume of medium.

The addition of 1% sodium formate to peptone did not increase the volume consistently or appreciably. In comparing gas volumes, too much significance must not be placed on fluctuations of a few cubic centimeters, as already emphasized on numerous occasions. However, 12 of the 19 strains showed an increased volume; in the remainder it was slightly decreased. Despite this rather inconclusive result, the gas constituents changed radically. The CO_2/H_2 ratio dropped decidedly, in some instances from numbers such as 33.6 to 4.2, 21.4 to 7.6, and so on. The actual numerical differences were not as marked with strains of *B. welchii*, but taking into consideration the nature of the fraction, the ratio was diminished from one-half to two-thirds of the value obtained in the plain peptone. The organism least sensitive to either sodium formate or sodium chloride was *B. centrosporogenes*.

The sodium chloride exerted a marked influence on the gas composition in the cultures of 14 different strains. Only 5 organisms showed an increase in gas volume, which is inconclusive evidence for the

general view that sodium chloride stimulates bacterial activity. Nevertheless, the gas ratio was lowered considerably in every instance. Again *B. centrosporogenes* showed the least change in gas composition, the ratio being numerically the same as in the tests with sodium formate. It is interesting to note that with this exception, the CO_2/H_2 ratio of sodium chloride is represented by larger figures than those obtained from sodium formate. A 1% salt solution in plain water is hypertonic and combined with the salt content of the peptone may be in part the factor responsible for the change in ratio. Authorities differ in their

TABLE 4
COMPARATIVE PEPTONE FERMENTATION AFTER 7 DAYS' INCUBATION

Organism	Strain	Plain Peptone		Peptone + 1% HCOONa		Peptone + 1% NaCl	
		Ratio $\frac{\text{CO}_2}{\text{H}_2}$	Standard Volume, Cc.	Ratio $\frac{\text{CO}_2}{\text{H}_2}$	Standard Volume, Cc.	Ratio $\frac{\text{CO}_2}{\text{H}_2}$	Standard Volume, Cc.
1. <i>B. botulinus</i>	97	6.0	70	3.58	78	4.4	90
2. <i>B. botulinus</i>	19	11.4	63	1.4	100
3. <i>B. botulinus</i>	62	25.9	120	5.4	100	6.1	107
4. <i>B. botulinus</i>	34	27.7	125	17.0	117
5. <i>B. botulinus</i>	53	5.2	46	1.35	35
6. <i>B. botulinus</i>	38	30.8	120	6.17	125	7.7	80
7. <i>B. botulinus</i>	26	21.4	130	7.6	135
8. <i>B. sporogenes</i>	46	25.6	118	4.9	135	7.1	130
9. <i>B. sporogenes</i>	114	18.3	163	5.9	175
10. <i>B. sporogenes</i>	Y	70.1	118	8.5	118	30.1	125
11. <i>B. sporogenes</i>	48	33.6	164	4.2	125	5.3	153
12. <i>B. welchii</i>	57	0.40	42	0.26	25
13. <i>B. welchii</i>	10/46	0.30	29	0.16	18
14. <i>B. welchii</i>	26	0.15	16	0.08	22	0.12	15.4
15. <i>B. welchii</i>	2	0.77	17	0.17	19	0.23	17.3
16. <i>B. welchii</i>	129	0.33	19	0.15	17	0.23	15
17. <i>B. welchii</i>	36	0.58	28	0.19	30	0.23	35
18. <i>B. welchii</i>	135	0.27	30	0.30	35	0.22	20
19. <i>Vibrio septique</i>	99-139	0.93	28	0.69	11
20. <i>B. centrosporogenes</i>	82	37.4	50	25.5	80	25.5	63
21. <i>B. bifementans</i>	62	45.1	46	8.7	40	11.4	35

views concerning the effect of osmotic pressure on aerobic growth. No references have been found which deal with the same phenomenon under anaerobic conditions.

The results obtained with sodium formate could be used as evidence to support the equation of Pakes and Jollyman.⁸ It is recalled that they concluded from their studies that this salt is decomposed into bicarbonate and hydrogen. The medium is hardly acid enough to decompose either the carbonate or the bicarbonate formed. The carbon dioxide found in the analyses is probably due to the peptone fermentation rather than the product of formate decomposition. The higher per-

centage of H_2 evolved in the presence of sodium chloride cannot be explained by a similar equation. This behavior indicates that the hypotheses which are used to explain reactions of salts in a complex organic peptone medium are questionable. In any fluid except a synthetic medium, in which other reactions can be carefully traced, the various hypothetical equations thus far published must be recognized as highly speculative. Miss E. Wagner in this laboratory found that the volatile acid fraction of *B. botulinus*, *B. sporogenes* and other anaerobes contained traces of formic acid. It is therefore not unlikely that small amounts of formic acid may be formed, but they are rapidly catabolized. Harden,² discussing the differences between *B. typhosus* and *B. coli communis* in their action on glucose, mentions that the former produces formic acid, while the latter gives CO_2 and H_2 . He states:

The particular *B. coli communis* employed in these experiments exerted comparatively little action on a solution of 2% sodium formate and 1% Witte peptone. In the presence of a small amount of sugar, however, a much larger amount of the formate was decomposed as shown in the following table, the hydrogen due to the sugar being deducted.

Sodium formate, per cent.....	2.0	2.0	2.0
Glucose, per cent.....	0.0	0.2	0.4
Hydrogen in c.c.....	105.0	1122.0	1481.0

The main advantage of adding the glucose is doubtless found in the increased acidity due to the fermentation. Some of the sodium formate added in excess, as for example 2%, is converted to the acid, which is more readily decomposed than the salt. It is doubtful whether the amount of H_2 due to the glucose could be accurately ascertained and deducted from the volume originating from the formate. This phase of the problem will be discussed in connection with the experiment on anaerobic fermentation in a glucose, formate, peptone medium. Great specificity of reactions should not be attributed to a single salt. It will be shown later that the addition of 1% sodium nitrate raised the H_2 content of the gas evolved from a 1% glucose, 2% peptone medium.

The general conclusions from table 4 are: (1) The addition of sodium formate and sodium chloride to a peptone medium does not appreciably alter the volume of gas from that obtained on plain peptone. (2) The sodium formate may be the source of the greater percentage of H_2 . However, it is reasonable to assume that the effect of sodium formate may have the same explanation as the changes observed by the addition of sodium chloride, namely, altered physical environment. (3) The addition of sodium chloride to a peptone medium increases the

percentage of H_2 . This increase in H_2 is due to: (a) a chemical participation of the sodium chloride in the reaction, for example, a saturation of double bond compounds in the metabolic products, (b) a change in the osmotic pressure and (c) a combination of the processes mentioned under (a) and (b).

Glucose Fermentation at the End of a 'Three Days' Incubation Period.—In Table 5A are shown the gas analysis records obtained from a 1% glucose, 2% peptone medium. The 3 gm. of glucose dissolved in 10 c.c. of water have been sterilized in the Arnold sterilizer and have been added to the stock 300 c.c. amount of autoclaved peptone solution. The error in the CO_2 fraction as estimated by the Hesse method is given in the last column of the table. The average error of +4.1% for 4 strains of *B. botulinus* and 3 cultures of *B. sporogenes* is higher than in the plain peptone solution. The total amount of gas evolved is also much higher than in the plain peptone medium. The process is much more active, and it is quite probable that more of the peptone is metabolized. The proteolytic organisms blacken the mediums, while the saccharolytic types give a light brownish color, although the same approximate volume of gas is evolved from the medium.

In a series of tests, the amount of sugar remaining in the medium has been determined by the Folin-McEllroy method.⁹ Sterile peptone, whether 0.5% or 2%, did not affect the end point of the reaction in this rapid copper method. The technic as originally applied to urine has been tested experimentally and found to be accurate to within 0.05% when applied to peptone solutions. The percentage of the carbohydrate utilized varied greatly with different strains. This variation is not in proportion to the amount of gas evolved. In every instance, except when 0.25% disodium phosphate has been added, less than 0.5% of the glucose is catabolized. When the phosphate buffer is used, as high as 0.7% glucose is decomposed. This is quite in contrast to the results secured when 1% sodium nitrate is added to the carbohydrate medium. The organisms apparently utilized a very small amount of glucose in proportion to the volume of gas evolved. This result may be due to other chemical groups (NO_2) exerting a reducing action on the copper sulphate solution of the Folin-McEllroy reagent, which in turn may give the erroneous impression of a large sugar residue. Reducing reactions in the presence of widely varied products of bacterial metabolism on a complex peptone are always open to suspicion. The time of reduc-

⁹ Jour. Biol. Chem., 1919, 38, p. 287.

tion and alkalinity of the solution have a marked influence on the end point of reaction. Although these factors can be standardized under known conditions, no confidence in the quantitative accuracy of the method is felt in the presence of interfering or reducing substances. Since the figures showed nothing which could be correlated with the gas analyses, they are omitted from the tables.

It can be seen from the data secured with 9 strains that *B. botulinus* does not give a constant CO_2/H_2 ratio. Five strains approximate a ratio of 4, while 2 strains have a numerical value around 10. Strain 40 is very peculiar in all mediums; it never gives more than a trace of H_2 . This strain fails to break the liver agar in shake tubes, but it produces a potent toxin.¹⁰ The constituent gases show no differences in composition which would differentiate the *B. botulinus* cultures, types A and B. Strains 26, 34, 36, 40 and 53 are immunologically type B, while the remainder are type A. Strain 97 produces spores which are the most heat resistant of any tested in this laboratory. It is interesting to note that its CO_2/H_2 ratio varies least when grown in the different mediums. This fact would indicate that the anaerobe has the faculty to adjust itself to the environment with little disturbance in the process of metabolism. There are no distinct differences in volume of gas, P_H change, appearance of the medium, or CO_2/H_2 ratio to distinguish *B. sporogenes* from *B. botulinus*. It is well known that these organisms are difficult to differentiate both culturally and morphologically. Here, as in plain peptone, *B. histolyticus* WV fails to evolve H_2 .

The strains of *B. welchii*¹¹ do not blacken the medium, and the P_H is more acid than with *B. botulinus* or *B. sporogenes*. Strain 129 produces a small volume of gas, and furthermore gives an exceptionally low gas ratio of 0.85; this ratio is held consistently in all types of medium. Keyes and Gillespie¹² obtained for *B. welchii* a ratio of 1.53 in a 1% Witte peptone, 1% "Merck" glucose solution incubated for 5 days and an average of 1.43 when grown for 14 days. However, the ratios calculated in the present series of tests approximate unity. *Vibrio septique*, as already mentioned by Kahn,¹³ exhibits characteristics of a

¹⁰ This strain has since become nontoxic, but the CO_2/H_2 ratio and the gas analytic behavior of the cultures have remained the same. This strain, together with a number of other *B.* types (26) which have changed from toxic to nontoxic strains in the course of prolonged artificial cultivation is now the subject of careful study in this laboratory.

¹¹ We are indebted to Ivan C. Hall, of the University of California for *B. welchii* strains 2, 26, 129, 36 and 135. Professor Hall has published several papers on these strains in *Jour. Infect. Dis.* (1922, 30, p. 445). In order to avoid confusion, his serial numbers have not been changed.

¹² *Jour. Biol. Chem.*, 1913, 13, p. 291.

¹³ *Jour. Med. Res.*, 1922, 43, p. 155.

TABLE 5
(A.) GLUCOSE FERMENTATION AFTER 3 DAYS' INCUBATION

Organism	Strain	Appearance of Medium	P _H Change	Volume at Standard, Cc.	CO ₂ %	H ₂ %	N ₂ %	Sum, %	Ratio CO ₂ / H ₂	Error in CO ₂ Fraction, %	Total Volume Ratio 7 day / 3 day Incubation
1. <i>B. botulinus</i> type A.....	97	Black	7.4-6.2	325	77.1	21.3	1.5	99.9	3.66	
2. <i>B. botulinus</i> type A.....	19	Black	7.4-6.4	385	79.4	19.3	1.4	100.0	4.14	
3. <i>B. botulinus</i> type A.....	62	Black	7.6-6.8	268	79.2	18.2	2.4	99.8	4.35	+3.7	
4. <i>B. botulinus</i> type B.....	34	Black	7.4-5.4	350	84.9	13.1	1.9	99.9	6.47	
5. <i>B. botulinus</i> type B.....	53	Black	7.4-6.0	435	76.9	19.9	3.1	99.9	3.86	+4.6	
6. <i>B. botulinus</i> type B.....	39	Black	7.4-6.2	440	77.5	20.5	1.8	99.8	3.78	
7. <i>B. botulinus</i> type A.....	38	Black	7.4-6.6	205	86.0	7.2	7.5	99.7	11.9	+5.0	
8. <i>B. botulinus</i> type B.....	26	Black	7.4-5.2	330	88.5	8.8	2.6	99.9	10.0	
9. <i>B. botulinus</i> type B.....	40	Black	7.4-6.4	300	97.5	0.9	1.4	99.1	+3.5	
10. <i>B. sporogenes</i>	46	Black	7.4-6.4	305	84.0	13.1	2.8	99.9	6.4	+4.1	
11. <i>B. sporogenes</i>	114	Black	7.4-6.2	300	86.5	11.7	1.6	99.8	7.3	+4.6	
12. <i>B. sporogenes</i>	Y	Black	7.4-5.6	550	76.0	22.7	1.1	99.8	3.35	
13. <i>B. sporogenes</i>	48	Black	7.6-6.2	425	69.0	27.3	3.3	99.9	2.5	+3.1	
14. <i>B. sporogenes</i>	6	Black	7.4-5.7	360	87.3	10.6	2.2	100.1	8.2		
15. <i>B. sporogenes</i>	3	Black	7.4-5.7	350	80.1	18.8	1.1	100.0	4.5		
16. <i>B. sporogenes</i>	2	Black	7.4-5.8	300	85.8	11.9	2.2	99.9	7.2		
										+4.1*	
17. <i>B. welchii</i>	57	Brown	7.4-5.4	240	49.4	50.0	0.6	100.0	0.99		
18. <i>B. welchii</i>	10/46	Brown	7.4-5.4	245	52.0	47.0	1.1	100.1	1.1		
19. <i>B. welchii</i>	26	Brown	7.4-4.6	310	50.3	47.8	1.7	99.8	1.05		
20. <i>B. welchii</i>	2	Brown	7.4-5.0	410	49.1	49.0	1.5	99.6	1.0		
21. <i>B. welchii</i>	129	Brown	7.4-5.2	195	45.2	53.1	1.7	100.0	0.85		
22. <i>B. welchii</i>	36	Brown	7.4-4.2	330	48.7	48.5	2.6	99.8	1.0		
23. <i>B. welchii</i>	135	Brown	7.4-4.2	320	48.8	49.0	2.0	99.8	0.99		
24. <i>Vibrio septique</i>	RNS	Brown	7.4-4.2	60	49.8	47.5	2.7	100.0	1.05		
25. <i>Vibrio septique</i>	99-139	Brown	7.4-4.4	150	47.2	51.0	1.7	99.9	0.93		
26. <i>B. centrosporogenes</i>	82	Brown	7.6-6.0	300	64.0	30.5	4.9	99.4	2.1		
27. <i>B. histolyticus</i> ...	WV	Black	7.4-7.0	67	88.5	0.0	11.4	99.9		
28. <i>B. bifermentans</i> .	62	Brown	7.4-6.0	75	57.0	34.2	8.9	100.1	1.66		

(B.) GLUCOSE FERMENTATION AFTER 7 DAYS' INCUBATION

1. <i>B. botulinus</i>	97	Black	7.4-5.6	510	75.5	24.0	0.7	100.2	3.1	+2.5	1.6
2. <i>B. botulinus</i>	19	Black	7.4-5.0	618	73.5	21.9	4.2	99.6	3.36	+3.2	1.6
3. <i>B. botulinus</i>	62	Black	7.4-5.4	327	74.5	30.3	5.0	99.8	3.66	+3.0	1.2
4. <i>B. botulinus</i>	53	Black	7.4-5.8	630	73.6	21.1	4.2	98.9	3.5	+2.6	1.5
5. <i>B. botulinus</i>	38	Black	7.4-5.6	282	83.0	15.1	1.8	99.9	5.5	+3.9	1.6
6. <i>B. botulinus</i>	40	Black	7.4-6.0	467	95.2	0.0	4.7	99.9	+4.7	1.6
7. <i>B. sporogenes</i>	46	Black	7.4-5.6	430	82.2	16.8	0.9	99.9	4.9	+3.4	1.4
8. <i>B. sporogenes</i>	114	Black	7.4-6.0	560	83.6	13.5	2.5	99.6	6.2	+3.9	1.9
9. <i>B. sporogenes</i>	48	Black	7.4-5.4	610	64.5	21.2	14.1	99.8	3.0	+1.8	1.5
										+3.2*	
10. <i>B. welchii</i>	57	Brown	7.4-4.8	645	42.3	39.2	18.0	99.5	1.08	2.6
11. <i>B. welchii</i>	10/46	Brown	7.4-4.8	460	39.3	38.4	22.1	99.8	1.02	1.9
12. <i>B. welchii</i>	36	Brown	7.4-5.0	650	46.0	52.0	2.0	100.0	0.89	1.9
13. <i>B. welchii</i>	135	Brown	7.4-4.0	550	47.8	50.8	1.2	99.8	0.94	1.8
14. <i>B. centrosporogenes</i>	82	Brown	7.4-5.2	370	62.0	35.7	2.0	99.7	1.74	1.2
15. <i>B. histolyticus</i> ...	WV	Black	7.4-7.0	63	90.7	0.99	7.9	99.6	1.0
16. <i>B. bifermentans</i> .	62	Brown	7.4-5.6	150	46.5	36.0	17.5	100.0	1.29	2.0
17. <i>B. tetani</i>	67	Black	7.4-7.2	20	42.7	43.7	13.4	99.8	0.98		
18. <i>B. tetani</i>	VT5p.	Black	7.4-7.2	17	47.0	34.4	17.9	99.3	1.36		

* Average.

TABLE 5—Continued
(C.) SALICIN FERMENTATION AFTER 3 DAYS' INCUBATION

Organism	Strain	Ap- pear- ance of Medium	P _H Change	Volume at Stan- dard, C.	CO ₂ %	H ₂ %	N ₂ %	Sum, %	Ratio CO ₂ — H ₂
1. <i>B. botulinus</i>	97	Black	7.4-6.4	270	75.8	22.8	1.4	100.0	3.32
2. <i>B. botulinus</i>	19	Black	7.4-6.4	325	72.6	25.2	2.1	99.9	2.88
3. <i>B. botulinus</i>	62	Black	7.4-6.1	400	69.9	23.8	5.9	99.6	2.94
4. <i>B. botulinus</i>	34	Black	7.4-5.8	325	86.0	11.9	2.0	99.9	7.23
5. <i>B. botulinus</i>	53	Black	7.4-6.6	300	72.9	25.8	1.2	99.9	2.82
6. <i>B. botulinus</i>	39	Black	7.4-6.4	320	79.6	18.4	1.8	99.8	4.35
7. <i>B. botulinus</i>	38	Black	7.4-6.7	236	83.7	8.3	7.7	99.7	10.1
8. <i>B. botulinus</i>	26	Black	7.4-6.4	275	84.8	12.8	2.1	99.7	6.6
9. <i>B. sporogenes</i>	46	Black	7.4-6.7	178	93.3	0.7	5.5	99.5	130.0
10. <i>B. sporogenes</i>	114	Black	7.4-6.3	165	80.5	15.7	3.8	100.0	5.1
11. <i>B. sporogenes</i>	Y	Black	7.4-6.4	210	90.2	8.8	1.0	100.0	10.3
12. <i>B. sporogenes</i>	48	Black	7.4-6.4	215	88.5	5.5	5.7	99.7	16.2
13. <i>B. sporogenes</i>	6	Black	7.4-6.4	225	94.1	2.9	2.8	99.8	32.5
14. <i>B. sporogenes</i>	3	Black	7.4-6.8	75	85.0	11.0	4.0	100.0	7.73
16. <i>B. sporogenes</i>	2	Black	7.4-6.7	75	85.6	11.9	2.4	99.9	7.2
17. <i>B. welchii</i>	57	Brown	7.4-7.2	21	18.8	67.1	12.7	99.6	0.28
18. <i>B. welchii</i>	10/46	Brown	7.4-5.6	325	48.8	49.8	1.3	99.9	0.98
19. <i>B. welchii</i>	26	Brown	7.4-5.6	115	40.5	55.5	3.8	99.8	0.73
20. <i>B. welchii</i>	2	Brown	7.4-6.5	140	43.8	48.7	7.5	100.0	0.9
21. <i>B. welchii</i>	36	Brown	7.4-7.2	22	7.7	56.1	36.0	99.8	0.14
22. <i>B. welchii</i>	135	Brown	7.4-7.3	27	20.6	67.2	12.2	100.0	0.31
23. <i>Vibrio septique</i>	RNS	Yellow	7.4-7.1	35	94.0	0.0	6.0	100.0
24. <i>Vibrio septique</i>	99-139	Yellow	7.4-4.2	175	48.4	50.8	0.7	99.9	0.96
25. <i>B. bifementans</i>	62	Yellow	7.4-6.6	62	93.0	5.0	1.5	99.5	18.6

purely saccharolytic organism; the value for the CO₂/H₂ ratio is around unity. The volume of gas is much less than that produced by *B. welchii* strains, but the P_H change is just as great as in the case of the greater gas producer. *B. bifementans* is considered by Kahn feebly proteolytic, and the higher CO₂/H₂ ratio of 1.66 in the present series would indicate a slight degree of proteolysis.

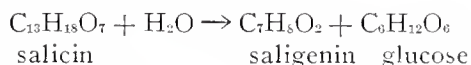
Glucose Fermentation at the End of a Seven Days' Incubation Period.—It is evident from the data in Table 5B that the volumes of gas formed by *B. sporogenes* and *B. botulinus* in a 1% glucose, 2% peptone medium, incubated for 7 days, are approximately 1.6 times larger than those obtained from the cultures at the end of 3 days. This fact indicates that the process is more active during the first 3 days of incubation, although the sugar analysis revealed an unused portion of at least 0.5% glucose. The saccharolytic organisms continue to use the glucose at about the same rate and give a volume twice as great for the longer incubation period. The average error in the CO₂ fraction, according to the Hesse method is lower after the 4 additional days' incubation. There obviously is no doubt that the foreign gases in the

CO₂ are formed in greater quantities during the first 3 days. The error in the CO₂ fraction is slightly less than that found in the plain peptone during the same incubation period. *B. histolyticus* forms about 1% hydrogen, which indicates that on prolonged incubation it utilizes some of the sugar.

Attention is called to analyses 17 and 18 dealing with *B. tetani*. The volumes of gas evolved during one week of growth in the rich medium of this experiment are too small to justify analyses of cultures in mediums of other composition or grown for shorter periods. In ordinary qualitative tests, the incubation period is usually short, and *B. tetani* is recorded as a nonfermenter of all carbohydrates. Peptone is broken down with the formation of H₂S, as indicated by the blackening of the fluid. Kahn¹³ classes this organism as feebly proteolytic. Wagner, Dozier and Meyer,¹⁴ when making volatile acid determinations on cultures of *B. tetani* in peptic digest broth, found a large fraction of acids of low molecular weight. This organism is apparently unable to utilize even the simplest organic acids, such as formic or acetic acid, or to transform them into gas during the first 2 weeks of incubation. Based on studies of the nitrogen metabolism made on the 3rd to the 18th day of cultivation, the organism is considered more highly proteolytic than *B. sporogenes* or *B. botulinus*. Amino acid formation during the same period does not indicate such a high degree of proteolysis as would be assumed by the slow but high ammonia production.

The total volume of CO₂ and H₂ evolved per 100 c.c. of medium has been computed. With the exception of *B. welchii*, the volume of H₂ is not proportionate to the amount of carbohydrate utilized, as determined by the Folin-McEllory method. This observation shows that side reactions occur in the medium, especially with the proteolytic organisms. The CO₂/H₂ ratios will be discussed later and compared with those of the 3 day period.

Salicin Fermentation at the End of a Three Days' Incubation Period.—Salicin is not a true sugar, although often spoken of as such by the bacteriologist. Chemically it is known as a glucoside; its natural source is the bark and the leaves of the willow tree (*salix*), from which it derives its name. Salicin may be chemically hydrolyzed into saligenin and glucose according to the reaction:



¹⁴ Jour. Infect. Dis., 1924, 34, p. 63.

This reaction may represent the manner by which salicin fermenting bacteria utilizes the compound. The resulting glucose serves as the source for gas production. If the glucoside is split by bacteria according to the foregoing equation, the reaction of hydrolysis and the breakdown into gas occurs in small quantities, and the two reactions take place simultaneously. Numerous tests for a reducing sugar with Benedict's reagent gave negative results even when the salicin fermentation had been in progress vigorously for 3 days. All strains of *B. botulinus* studied in this laboratory are salicin fermenters. The majority of *B. sporogenes* strains show no gas qualitatively in the course of 2 days' incubation, as proved by the unpublished work of Mrs. K. Oman Hoskins and by Hall.¹⁵ Strains of *B. welchii* are not infrequently salicin fermenters, as seen from the preceding table 5C.¹⁶

In judging from the gas analyses whether a strain is a positive fermenter or not, two conditions must be remembered: (1) the volume of gas, (2) the percentage of H_2 . The hydrogen on account of its insolubility is seen qualitatively in Durham vials or Smith tubes, while the other fraction of the fermentation gases (CO_2) diffuses out of the mediums into the air. By comparing the data secured on a medium with 1% salicin and 2% peptone with those on plain peptone (table 3A), the direct influence of salicin on the fermentation can be established irrespective of the fact that the plain peptone has been incubated 4 days longer. All strains of *B. botulinus* and *B. sporogenes* formed much larger volumes of gas in the presence of salicin than without it. The salicin is apparently stimulating to *B. sporogenes*, although the relative percentages of H_2 are smaller than on the addition of glucose to the basic peptone medium. *B. sporogenes* 46 is the only strain which gives a lower percentage of H_2 than in plain peptone. This statement is supported by the CO_2/H_2 ratio. Strains 57, 36 and 135 of *B. welchii* give less volume in a salicin medium (3 days) than in a plain peptone solution incubated for 7 days. The gas production of *Vibrio septique* and *B. bifermentans* is not stimulated by salicin, with exception of one strain of *Vibrio septique* (99-139) which presented a large volume and a high percentage of H_2 . A comparison of the CO_2/H_2 ratios calculated from the data secured in mediums with the addition of either salicin or glucose to 2% peptone medium will be made in the next table. In general, the effect of 1% salicin can be considered as stimulating to

¹⁵ J. Infect. Dis., 1922, 30, p. 482.

¹⁶ Ibid., p. 461.

the anaerobes. This effect expresses itself in the production of a large volume of gas and an increased production of H_2 . The fermentation of the glucoside by *B. sporogenes* is, however, not as vigorous as in the presence of glucose.

Comparison of the Fermentation Reactions of Two Carbohydrates.

—In table 6, the data dealing with the fermentation of glucose and of salicin by various anaerobes after 3 days' incubation are detailed. The records of the results noted after a longer 7 day incubation period, on the constituent gases liberated from a 1% glucose, 2% peptone medium are included.

The volume of gas evolved by most of the strains of *B. botulinus* during 3 days' growth in the presence of 1% glucose is slightly smaller when salicin is employed. The CO_2/H_2 ratio in all except strains 34 and 39 is slightly lower; with these 2 strains it is a trifle higher. This change in ratio of these 2 strains indicates a depressed H_2 production. In contrast to *B. botulinus*, *B. sporogenes* evolves only half the amount of gas with salicin as with glucose. The gas ratio is much higher in 5 of the 7 strains. The lowered degree of H_2 production explains the observation that the qualitative fermentation reactions with salicin are usually negative. *B. sporogenes* 2 gives the same ratio in both glucose and salicin, but in the latter the gas volume is exactly one-fourth of that obtained in glucose. These conditions may lessen the gas volume in the qualitative tests and lead to inconclusive results. *B. sporogenes* 114 gives one-half the volume of gas in salicin and a gas ratio lower than that in glucose. A qualitative test would undoubtedly prove positive in from 24 to 48 hours. The gas evolved by strain 114 in the salicin medium was analyzed on 3 different occasions. A freshly isolated culture was used with each duplicate analysis in order to assure its purity. It is interesting to note that the organisms shows a mutation of colony formation in deep liver agar shakes; the wooly fluff is changed to tufted disks. All transplants from the seed cultures gave good wooly colonies in 24 hours; they were used for the inoculation of the flasks. The 3 check analyses with strain 114 made on mediums with 1% salicin are presented in table 2. It will be seen that salicin is to a certain degree utilized by *B. sporogenes*; the gas volume is increased, but the CO_2/H_2 ratio is lower than in plain peptone. Comparing salicin fermentation with that of glucose, it is concluded that the former generates less volume and the gas contains a smaller percentage of H_2 . This statement holds true for all the strains of *B. sporogenes* tested except 114.

Three strains of *B. welchii*, 10/46, 26 and 2, are salicin fermenters. They give a good volume of gas and the gas ratio varies from 0.73 to 0.98, which is only slightly lower than that secured in glucose. Strain 10/46 gives regularly a large amount of gas in salicin, as shown in table 2, in which duplicate analyses are recorded. The other strains of *B. welchii* form the same amount of gas, but a lower ratio than in plain peptone. *Vibrio septique* RNS is a salicin nonfermenter, but strain

TABLE 6
COMPARATIVE CARBOHYDRATE PEPTONE FERMENTATION

Organism	Strain	I 1% Glucose Incubated Seven Days		II 1% Glucose Incubated Three Days		III 1% Salicin Incubated Three Days	
		Ratio CO ₂	Stan- dard Volume, Cc.	Ratio CO ₂	Stan- dard Volume, Cc.	Ratio CO ₂	Stan- dard Volume, Cc.
		H ₂		H ₂		H ₂	
1. <i>B. botulinus</i>	97	3.1	510	3.66	325	3.32	270
2. <i>B. botulinus</i>	19	3.36	618	4.14	385	2.88	325
3. <i>B. botulinus</i>	62	3.66	327	4.35	268	2.94	400
4. <i>B. botulinus</i>	34	6.47	350	7.23	325
5. <i>B. botulinus</i>	53	3.5	630	3.86	435	2.82	300
6. <i>B. botulinus</i>	39	3.78	440	4.33	320
7. <i>B. botulinus</i>	38	5.5	282	11.9	205	10.1	236
8. <i>B. botulinus</i>	26	10.0	330	6.6	275
9. <i>B. botulinus</i>	40	No H ₂	467	No H ₂	300
10. <i>B. sporogenes</i>	46	4.9	430	6.4	305	130.0	178
11. <i>B. sporogenes</i>	114	6.2	560	7.3	300	5.1	165
12. <i>B. sporogenes</i>	Y	3.35	550	10.3	210
13. <i>B. sporogenes</i>	48	3.0	610	2.5	425	16.2	215
14. <i>B. sporogenes</i>	6	8.2	360	32.5	225
15. <i>B. sporogenes</i>	3	4.3	350	7.73	75
16. <i>B. sporogenes</i>	2	7.2	300	7.2	75
17. <i>B. welchii</i>	57	1.08	645	0.99	240	0.28	21
18. <i>B. welchii</i>	10/46	1.02	460	1.1	245	0.98	325
19. <i>B. welchii</i>	26	1.05	310	0.73	115
20. <i>B. welchii</i>	2	1.0	410	0.9	140
21. <i>B. welchii</i>	36	0.89	650	1.0	330	0.14	22
22. <i>B. welchii</i>	135	0.94	550	0.99	320	0.31	27
23. <i>Vibrio septique</i>	RNS	1.05	60	No H ₂	35
24. <i>Vibrio septique</i>	99-139	0.93	50	0.96	175
25. <i>B. centrosporogenes</i>	82	1.74	370	2.1	300
26. <i>B. histolyticus</i>	WV	91+	63	No H ₂	67
27. <i>B. bifermentans</i>	62	1.29	150	1.66	75	18.6	62

99-139 is a marked salicin fermenter. The latter culture produces 3 times the volume of gas, but the ratio is approximately the same in salicin as in glucose. *B. bifermentans* is a salicin nonfermenter; the CO₂/H₂ ratio increases from 1.66 in glucose to 18.6, with little change in volume of gas.

Attention has already been called to the proportionate relationship of the volumes of gas evolved by the different bacteria after 3 and 7 day incubation periods. After prolonged incubation, the CO₂/H₂ ratios are

lower with the exception of *B. sporogenes* 48, which is numerically 0.5 higher. This striking tendency for a change in ratio of the constituent gases may be due to the following factors: (1) the constituent gases are not evolved at an equal rate throughout the fermentation, or (2) if they are formed at the same rate, the H_2 is partially absorbed in some side reaction which comes into play during the first few days of greatest physiologic activity. The latter explanation seems the most probable, both from the data presented in this paper and from deaminization noted by Harden.² This same change in the CO_2/H_2 ratio occurs when the medium is buffered by the addition of 1/4% disodium phosphate.

The observations dealing with a lower CO_2/H_2 ratio due to longer incubation are not entirely verified by the recent work of Bushnell.¹⁷ In order to discuss intelligently this aspect of the problem, it appeared advisable to retabulate his analytic results in accordance with the general principle followed in this paper. In 11 different kinds of mediums, he found more CO_2 on prolonged incubation. Based on complete biochemical findings, he identified the anaerobe employed in the study as *B. sporogenes*. His organism gave a rather low CO_2/H_2 ratio of 1.85 after 3 days' incubation in a 2% peptone, 0.5% glucose medium, while, as previously emphasized, an average ratio of 5.6 was characteristic for 7 strains of *B. sporogenes* studied by me in a broth with an excess of carbohydrate but incubated for the same period. It is impossible that the excess of 0.5% glucose should in itself be responsible for the differences in the ratios. His strain is a decided salicin fermenter. The gas analyses reported by Bushnell were made with a single strain, and the results are characteristic for this particular micro-organism. On the other hand, the statements and the conclusions of the present investigation are based on records secured with 24 different anaerobic strains of various species. A higher percentage of H_2 noted in cultures after prolonged incubation is obviously not characteristic of any species or type, but a process common to anaerobic fermentation.

Modifications of Glucose Fermentation by the Addition of Buffers, Nitrates, etc.—Keyes and Gillespie¹² report the effect of varying amounts of disodium phosphate and ammonium nitrate on the fermentation of *B. coli* in a 1% glucose, 1% ammonium lactate medium. The phosphate increased the gas volume and more CO_2 was evolved, raising the CO_2/H_2 ratio. In connection with their studies on the influence of inorganic salts, these workers were prompted to make the significant

¹⁷ Jour. Bacteriol., 1922, 7, p. 373.

statement that: "The effect of nitrate is to decrease slightly the carbon dioxide formation and use up most (or all) of the hydrogen that would otherwise be produced."

In the present study, the effect of disodium phosphate and sodium nitrate on 2% peptone, 1% glucose medium has been investigated. In a

TABLE 7
GAS PRODUCTION BY A CULTURE OF *B. SPOROGENES* ON DIFFERENT MEDIUMS ACCORDING TO
DATA OF L. D. BUSHNELL

Medium	Incu- bation, Days	CO ₂ %	H ₂ %	N ₂ %	Ratio CO ₂ H ₂	Standard Volume, Cc.
I						
2% peptone.....	3	72.7	17.3	10.0	4.2	43
	5	76.5	15.2	8.3	5.03	61
		81.7	12.6	5.7	6.5	77
	11	86.2	5.2	8.6	16.6	102
	14	86.1	8.9	5.0	9.7*	110
II						
2% peptone + 0.5% glucose....	3	62.2	33.7	4.1	1.85	200
	6	68.4	27.3	4.3	2.5	312
	10	90.0	333
III						
2% peptone + 1% lactose.....	2	75.9	16.9	7.2	4.5	82
	6	84.5	8.8	6.7	9.6	128
	9	88.2	8.6	3.2	10.2	133
IV						
2% peptone + 1% sucrose.....	7	18.3	70.9	10.8	0.258	28
	9	52.4	35.4	12.2	1.48	42
V						
2% peptone + 1% salicin.....	7	61.9	34.1	4.0	1.81	208
	9	65.8	30.7	3.5	2.14	228
VI						
2% peptone + 1% mannitol....	2	65.3	27.2	7.5	2.4	99
	9	85.0	10.8	4.2	7.86	137
VII						
2% peptone + 1% glycerol.....	7	46.5	44.5	9.0	1.04	227
	9	45.4	42.8	11.8	1.06	255
VIII						
2% peptone + 1% sol. starch...	4	44.1	53.6	2.3	0.82	18
	6	73.6	23.5	2.9	3.12	113
	9	71.2	24.7	4.1	2.88*	213
	12	79.2	18.0	2.7	4.4	316
IX						
2% peptone + 1% inulin.....	4	20.6	28
	6	29.3	70.0	0.7	0.42	30
	10	42.4	50.6	7.0	0.838	33
X						
Whole milk.....	5	76.8	15.0	8.2	5.1	...
	8	78.2	17.5	4.3	4.47*	...
XI						
Alkaline egg.....	2	64.9	26.4	8.7	2.46	66
	5	71.3	17.8	10.8	4.0	77
	8	70.1	23.9	6.0	2.94*	102
	12	73.7	16.8	9.5	4.38	112

* Only in these places does the CO₂ : H₂ ratio drop on longer incubation.

separate experiment the peptone content has been lowered to 0.5%, but the amount of glucose remained the same. The low peptone concentration is apparently the simplest source of nitrogen available for anaerobic fermentation. In such a medium, the glucose decomposition can be traced more closely as the side reactions are less intense, and the absorp-

tion of the gases by intermediary split products is diminished. The quantity of amino groups is reduced, and the retention of H_2 as suggested by Harden² or Neuberg⁴ is not as great as in the presence of 2% peptone.

Glucose Decomposition in the Presence of 0.5% Peptone.—The data presented in table 8A deal with the anaerobic fermentation in a medium composed of 1% glucose and 0.5% peptone after 3 days' incubation. The absence of a strong H_2S production does not indicate a scanty growth, but a lack of sulphur containing nitrogenous material. With the exception of the strain 38, the P_H change of the *B. botulinus* cultures is lower than in the stronger peptone, which acts as a buffer (table 5A). However, the total P_H change in all the cultures is about the same, showing the abundant acid formation from the glucose. On the other hand, the volumes of gas are greatly diminished. The gas volume of the saccharolytic organisms is neither as large in the nitrogen-poor as that produced in the nitrogen-rich medium (table 5A), nor is as great as that evolved by the proteolytic organisms in the 0.5% peptone broth. However, the CO_2/H_2 is always much lower than in the more concentrated peptone. This fact strongly suggests that the absorption of the H_2 by unsaturated peptone decomposition products is less active. The diminished utilization of the available carbohydrate explains furthermore the lower gas volumes, but it must be remembered that the bacteria were deprived of the necessary food and are mainly dependent on the sugar for their catabolic processes. *B. botulinus* 38 and 26 give gas ratios which are higher than those produced by other strains. This behavior is peculiar and constant in all mediums. It is noteworthy that the highly proteolytic *B. histolyticus* WV forms a fair amount of H_2 in a weak peptone solution. When deprived of nitrogenous nourishment, the microbe is apparently capable of slight saccharolytic activities giving a gas ratio of 11.6 (usual ratio >100). In referring to table 5A and 8A for a comparison, the following is noted: The difference in the composition of the gaseous end products indicates decided alterations in the metabolic processes of the organisms in 0.5% peptone solution. The glucose is probably the main source of the higher amounts of H_2 . *B. welchii* strains give unequal volumes of H_2 and CO_2 (47% to 62% H_2), which would indicate that even this highly saccharalytic organism does not yield gaseous end products according to the glucose decomposition equation of Harden.²

Effect of a Phosphate Buffer on Glucose Fermentation.—The medium employed to obtain the data in table 8B consisted of 1% glucose, 2% peptone and 0.25% disodium phosphate. This small percentage buffer did not alter the initial P_H . The phosphate solution was sterilized in the Arnold sterilizer and was added simultaneously with the glucose. The volumes of gas were much greater than those secured for the same incubation period (3 days) without the phosphate. It is interesting to note that the final P_H was generally lower than that recorded in table 5A. The phosphate delays the effect of the acids, and when the buffer was finally exhausted, growth continued vigorously. *B. histolyticus* formed a trace of H_2 , although none was found in the absence of phosphate. With the exception of *B. botulinus* 62, 53 and *B. centrosporogenes* 82, the CO_2/H_2 ratios were lower than those recorded in table 5A. The strains mentioned gave only slightly higher ratios. The same tendency in the ratio is observed in case the incubation period is lengthened from 3 to 7 days. Finally, it must be emphasized that the results here recorded are not in agreement with those of Keyes and Gillespie,¹² who found an increased yield of CO_2 due to phosphate in an ammonium lactate medium fermented anaerobically by *B. coli*.

Effect of Sodium Nitrate on Glucose Fermentation.—The gas analyses obtained from a medium composed of 1% sodium nitrate sterilized and added to the glucose-peptone solution in the usual manner, are given in table 8C. The P_H changes in the cultures are about the same as without the nitrate. Eighteen of 20 strains give less gas, probably due to the depressing effect of the nitrate. The same effect is produced by the addition of only 0.5% nitrate. The CO_2/H_2 ratio of proteolytic organisms is slightly lower than in the plain glucose-peptone fermentation. However, the most striking effect of the nitrate is recorded by the enormous increase in the CO_2/H_2 ratio with strains of *B. welchii*. The percentage of H_2 drops approximately from 50% to 2% in the presence of nitrate. *Vibrio septique* strains exhibit a similar tendency, although the percentage decrease in H_2 is not as great. This phenomena was first noticed by Pakes and Jollyman,¹⁸ while analyzing the gases produced by *B. coli* grown in a peptone-meat extract medium containing both 1% sodium formate and 1% potassium nitrate. This medium without nitrate gave a good volume of gas with approximately 50% hydrogen. The addition of nitrate lessened the gas volume greatly, and only traces of H_2 were found. *B. coli* is known to reduce nitrates

¹⁸ Jour. Chem. Soc. Trans., 1901, 79, p. 459.

TABLE 8

(A.) EFFECT OF LOW PEPTONE (0.5%) CONCENTRATION ON GLUCOSE (1%) FERMENTATION AFTER 3 DAYS' INCUBATION

Organism	Strain	Appearance of Medium	P _H Change	Volume at Standard, Cc.	CO ₂ %	H ₂ %	N ₂ %	Sum, %	Ratio CO ₂ / H ₂
1. B. botulinus.....	97	Yellow	7.4-5.2	215	60.4	38.6	1.0	100.0	1.56
2. B. botulinus.....	19	Yellow	7.4-5.6	138	52.2	44.5	3.0	99.7	1.17
3. B. botulinus.....	62	Yellow	7.6-6.3	69	64.6	32.6	2.7	99.9	1.98
4. B. botulinus.....	53	Yellow	7.6-5.4	200	56.6	41.7	1.7	100.0	1.36
5. B. botulinus.....	38	Yellow	7.4-7.0	70	69.0	24.8	6.1	99.9	2.78
6. B. botulinus.....	26	Yellow	7.6-5.6	110	67.3	29.4	3.4	100.1	2.28
7. B. botulinus.....	40	Yellow	7.6-5.8	60	96.0	0.33	3.46	99.7
8. B. sporogenes.....	46	Yellow	7.6-6.4	127	70.2	29.0	0.7	99.9	2.42
9. B. sporogenes.....	114	Yellow	7.6-6.2	132	62.5	35.1	2.4	99.9	1.79
10. B. sporogenes.....	48	Yellow	7.6-6.0	130	56.2	41.5	2.1	99.8	1.35
11. B. welchii.....	57	Yellow	7.6-4.4	98	38.4	58.7	2.9	100.0	0.66
12. B. welchii.....	26	Yellow	7.6-7.2	21	24.2	56.4	19.2	99.8	0.43
13. B. welchii.....	129	Yellow	7.6-6.2	40	34.3	62.6	3.0	99.9	0.55
14. B. welchii.....	36	Yellow	7.4-4.2	150	42.0	55.7	2.4	100.1	0.76
15. B. welchii.....	135	Yellow	7.6-4.2	85	44.5	47.3	7.6	99.4	0.94
16. B. centrosporogenes.	82	Yellow	7.6-5.8	95	57.0	37.6	5.3	99.9	1.5
17. B. histolyticus.....	WV	Yellow	7.6-7.0	20	85.0	7.3	7.8	100.1	11.6
18. B. bifermentans.....	62	Yellow	7.6-6.2	80	51.0	45.0	3.8	99.8	1.13

(B.) EFFECT OF Na₂HPO₄ (¼%) ON GLUCOSE (1%) FERMENTATION AFTER 3 DAYS' INCUBATION

1. B. botulinus.....	97	Black	7.6-6.3	390	74.4	24.4	1.1	99.9	3.07
2. B. botulinus.....	19	Black	7.6-5.7	555	65.8	31.6	2.3	99.7	2.08
3. B. botulinus.....	62	Black	7.6-5.8	490	81.3	16.5	1.9	99.7	4.92
4. B. botulinus.....	53	Black	7.4-6.3	550	78.3	20.1	1.6	100.0	3.9
5. B. botulinus.....	38	Black	7.4-5.2	540	66.3	32.3	1.3	99.9	2.05
6. B. botulinus.....	40	Black	7.6-6.3	375	97.6	0.0	2.3	99.9
7. B. sporogenes.....	46	Black	7.6-5.6	440	66.0	31.6	2.2	99.8	2.08
8. B. sporogenes.....	114	Black	7.6-5.6	700	78.6	18.8	2.6	100.0	4.18
9. B. sporogenes.....	48	Black	7.6-5.3	725	68.1	30.1	1.7	99.9	2.26
10. B. welchii.....	57	Yellow	7.4-4.2	405	45.3	52.5	2.2	100.0	0.86
11. B. welchii.....	10/46	Yellow	7.4-4.2	465	44.8	53.2	1.8	99.8	0.84
12. B. centrosporogenes.	82	Brown	7.4-5.4	650	67.0	29.4	3.4	99.8	2.28
13. B. histolyticus.....	WV	Brown	7.4-7.2	95	94.5	0.3	5.1	99.9
14. B. bifermentans.....	62	Yellow	7.4-5.4	90	47.5	50.0	2.5	100.0	0.95

(C.) EFFECT OF NaNO₃ (1%) ON GLUCOSE (1%) FERMENTATION AFTER 3 DAYS' INCUBATION

1. B. botulinus.....	97	Black	7.4-6.2	225	72.0	25.8	2.0	99.8	2.79
2. B. botulinus.....	19	Black	7.4-5.8	265	70.5	27.6	1.8	99.9	2.55
3. B. botulinus.....	62	Black	7.4-5.7	305	78.8	20.0	1.0	99.8	3.94
4. B. botulinus.....	53	Black	7.4-5.8	318	73.4	24.6	1.9	99.9	2.98
5. B. botulinus.....	38	Black	7.4-5.8	235	82.0	16.2	1.7	99.9	5.05
6. B. botulinus.....	26	Black	7.4-5.2	150	77.0	20.0	3.0	100.0	3.85
7. B. botulinus.....	40	Black	7.4-6.6	210	93.5	0.0	6.4	99.9
8. B. sporogenes.....	46	Black	7.4-5.8	445	82.5	15.8	1.5	99.8	5.2
9. B. sporogenes.....	114	Black	7.4-6.2	285	84.7	13.2	2.0	99.9	6.4
10. B. sporogenes.....	48	Black	7.4-6.6	165	69.9	26.2	3.8	99.9	2.66
11. B. welchii.....	57	Very yellow	7.4-6.6	182	88.3	2.9	8.7	99.9	30.0
12. B. welchii.....	10/46	Very yellow	7.4-6.3	250	89.5	1.5	8.9	99.9	60.0
13. B. welchii.....	26	Very yellow	7.4-5.6	70	82.0	2.8	15.1	99.9	29.3
14. B. welchii.....	2	Very yellow	7.4-5.8	100	91.8	2.5	5.5	99.8	36.7
15. B. welchii.....	129	Very yellow	7.4-6.3	51	92.0	3.1	4.9	100.0	29.6
16. B. welchii.....	36	Very yellow	7.4-5.6	160	90.0	1.9	7.9	99.8	90.0
17. B. welchii.....	135	Very yellow	7.4-5.6	150	89.2	1.0	9.8	100.0	89.2
18. B. centrosporogenes.	82	Black	7.4-5.6	110	75.5	22.5	2.0	100.0	3.3
19. B. histolyticus.....	WV	Black	7.4-6.4	154	96.3	0.46	3.1	99.9
20. B. bifermentans.....	62	Yellow	7.4-5.6	70	49.3	47.0	3.7	100.0	1.05
21. Vibrion septique.....	RNS	Yellow	7.4-5.8	23	68.0	16.0	15.5	99.5	4.25
22. Vibrion septique.....	99-139	Yellow	7.4-5.7	53	80.0	12.6	7.3	99.9	6.35

TABLE 8—*Continued*(D.) EFFECT OF NaNO_2 (1%) ON GLUCOSE (1%) FERMENTATION OF *B. WELCHII*

Organism	Strain	Appearance of Medium	P_{H} Change	Volume at Standard, Cc.	CO_2 %	H_2 %	N_2 %	Sum, %	Ratio $\frac{\text{CO}_2}{\text{H}_2}$
1. <i>B. welchii</i>	26	Deep yellow	7.4-7.2	38	28.6	7.2	64.0	99.8	3.97
2. <i>B. welchii</i>	2	Deep yellow	7.4-7.0	19	65.4	1.0	33.6	100.0	65.0
3. <i>B. welchii</i>	129	Deep yellow	7.4-7.0	15	10.9	1.3	87.8	100.0	10.5
4. <i>B. welchii</i>	36	Deep yellow	7.4-7.2	37	27.6	5.1	66.5	99.2	5.4
5. <i>B. welchii</i>	135	Deep yellow	7.4-7.1	23	38.3	3.0	58.3	99.6	12.8

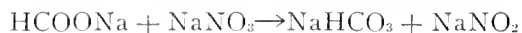
(E.) EFFECT OF SODIUM FORMATE (1%) ON 3 DAYS' GLUCOSE PEPTONE FERMENTATION

1. <i>B. botulinus</i>	97	Brownish	7.4-5.8	390	76.0	23.0	1.0	100.0	3.3
2. <i>B. botulinus</i>	53	Brownish	7.6-6.6	482	74.0	24.0	1.7	99.7	3.1
3. <i>B. sporogenes</i>	46	Brownish	7.4-5.7	420	77.3	19.6	3.0	99.9	3.95
4. <i>B. sporogenes</i>	6	Black	7.4-6.0	540	87.0	11.9	0.9	99.8	7.3

(F.) EFFECT OF SODIUM CHLORIDE (1%) ON 3 DAYS' GLUCOSE PEPTONE FERMENTATION

1. <i>B. botulinus</i>	97	Brownish	7.4-5.6	335	71.3	26.5	2.0	99.8	2.7
2. <i>B. sporogenes</i>	46	Brown	7.4-5.9	340	81.0	17.7	1.1	99.8	4.57
3. <i>B. sporogenes</i>	6	Black	7.4-5.6	375	81.6	16.3	2.0	99.9	5.0
4. <i>B. welchii</i>	129	Yellow	7.4-5.0	265	46.8	49.7	3.4	99.9	0.94

vigorously, and the oxygen so liberated is in turn reduced by the nascent hydrogen. They explain the oxidation by the equation:



This equation is based on the supposition that formates are decomposed into equal volumes of H_2 and CO_2 . In the presence of glucose, the nascent hydrogen evolved may act directly as the reducing agent without intermediate steps. In order to test this supposition, qualitative tests for nitrites (KI reduction) in the cultures of *B. welchii* have been made. Positive reactions have invariably been noted, but no quantitative determinations of the amount of nitrate reduced have thus far been made. In order to ascertain whether *B. welchii* is capable of further reducing the nitrite radical, 1% sodium nitrite has been added to the usual glucose-peptone medium. The required weight of nitrite is dissolved in water and sterilized by passage through a Berkefeld candle.

The chemical is mixed with the peptone solution shortly before the inoculation of the flask, to prevent oxidation. Samples of the nitrite medium stratified with petrolatum are tested for sterility in separate test tubes. The results of several gas analyses in such a medium are given in table 8D. The sodium nitrite proved very toxic; the gas ratios are entirely different than those recorded in previous experiments. The CO_2/H_2 ratios resemble those found in mediums with a 1% nitrate addition, but they have a lower percentage of carbon dioxide. It seems that although the sodium nitrite is highly depressing, it has a feeble oxidizing power on the H_2 evolved. The large quantity of residual nitrogen (30% to 65%) indicates strongly that the nitrite is further reduced to inert nitrogen by *B. welchii*. However, the actual number of cubic centimeters of nitrogen is small on account of the low total gas volume. At some future date, the intensity of the nitrate reduction will be determined by chemical analysis of the medium before and after growth of *B. welchii*. Apparently, the reduction phenomena is characteristic of both *B. coli* and *B. welchii*.

Effect of Sodium Formate and Sodium Chloride on Glucose Fermentation.—The addition of sodium formate (1%) changed decidedly the constituent gases evolved from a 2% peptone solution. The P_{H} in the plain peptone at the termination of the experiment was neutral. It is therefore doubtful whether appreciable amounts of the salt were converted into formic acid. The stimulating effect observed may be attributed to the sodium salt and not to the direct metabolism of the acid derivative. It will be recalled that sodium chloride provoked nearly the same result, although it failed to lower the CO_2/H_2 ratio in the same degree. The effect of both salts was studied with several strains of bacteria, in order to ascertain the degree of stimulation caused by the acid P_{H} in an active glucose fermentation.

The gas analyses of cultures prepared in a 2% peptone, 1% glucose medium to which 1% sterile HCOONa or NaCl have been added are given in tables 8E and F. The HCOONa stimulates the total gas production from 50 c.c. to 180 c.c. in different flasks. The volume formed by *B. sporogenes* is increased more markedly than that of *B. botulinus*. It is not as great (15 c.c. to 70 c.c.) in cultures containing NaCl . With the exception of *B. welchii* 129, the addition of these salts lowers the CO_2/H_2 ratios. *B. welchii* strain 129 gives a ratio of 0.09 higher in the presence of NaCl . The differential increase in hydrogen production as indicated by the CO_2/H_2 ratios cannot be defi-

nately determined on account of the small number of analyses. However, if the increase in gas volume is taken as an index of stimulation, the formate has a greater effect than the chloride. Harden² encountered larger quantities of H_2 in a formate medium (1% peptone and 2% sodium formate) enriched by glucose and fermented by *B. coli*. The fermentation of the glucose furnished the necessary acidity to convert the sodium salt into formic acid, which is the simplest known

TABLE 9
COMPARATIVE GLUCOSE FERMENTATION AFTER 3 DAYS' INCUBATION

Organism	Strain	I 2% Peptone 1% Glucose		II 2% Peptone 1% Glucose + 1% $NaNO_3$		III 2% Peptone 1% Glucose + ¼% Na_2HPO_4		IV 0.5% Peptone 1% Glucose	
		Ratio CO ₂	Stand- ard Vol- ume, Cc.	Ratio CO ₂	Stand- ard Vol- ume, Cc.	Ratio CO ₂	Stand- ard Vol- ume, Cc.	Ratio CO ₂	Stand- ard Vol- ume, Cc.
		H ₂		H ₂		H ₂		H ₂	
1. <i>B. botulinus</i>	97	3.66	325	2.79	225	3.07	390	1.56	215
2. <i>B. botulinus</i>	19	4.14	385	2.55	265	2.08	555	1.17	138
3. <i>B. botulinus</i>	62	4.35	268	3.94	305	4.92	490	1.98	69
4. <i>B. botulinus</i>	53	3.86	435	2.98	318	3.9	550	1.36	200
5. <i>B. botulinus</i>	38	11.9	205	5.05	235	2.05	540	2.78	70
6. <i>B. botulinus</i>	26	10.0	330	3.85	150	2.28	110
7. <i>B. botulinus</i>	40	No H ₂	300	No H ₂	210	No H ₂	375	(0.33% H ₂)	60
8. <i>B. sporogenes</i>	46	6.4	305	5.2	445	2.08	440	2.42	127
9. <i>B. sporogenes</i>	114	7.3	300	6.4	285	4.18	700	1.79	132
10. <i>B. sporogenes</i>	48	2.5	425	2.66	165	2.26	725	1.35	130
11. <i>B. welchii</i>	57	0.99	240	30.0	182	0.86	405	0.66	98
12. <i>B. welchii</i>	10/46	1.1	245	60.0	250	0.84	465
13. <i>B. welchii</i>	26	1.05	310	29.3	70	0.43	21
14. <i>B. welchii</i>	2	1.0	410	36.7	100
15. <i>B. welchii</i>	129	0.85	195	29.6	51	0.55	40
16. <i>B. welchii</i>	56	1.0	330	90.0	160	0.76	150
17. <i>B. welchii</i>	135	0.99	320	89.2	150	0.94	85
18. <i>B. centrosporogenes</i>	82	2.1	300	3.3	110	2.28	650	1.5	95
19. <i>B. histolyticus</i>	WV	No H ₂	67	No H ₂	154	No H ₂	95	11.6	20
20. <i>B. bifermentans</i>	62	1.66	75	1.05	70	0.95	90	1.13	80
21. <i>Vibrio septique</i>	RNS	1.05	60	4.25	23
22. <i>Vibrio septique</i>	99-139	0.93	50	6.35	53

organic acid. It is reasonable to assume that the formic acid is decomposed to some extent into CO_2 and H_2 in an acid environment. The same reasoning does not apply to the reaction with sodium chloride. However, it may explain the growth-promoting properties of formate in contrast to the lower degree of activity exerted by the chloride.

Comparison of Modified Glucose Fermentation.—In order to discuss more clearly the influence of lowering the peptone content, the addition of buffer, and sodium nitrate, etc., the detailed data are condensed in table 9. It is obvious that the experimental conditions were uniform

throughout. In columns I, II and III, the glucose and peptone content has been kept constant; in columns II and III, inorganic salts have been added. In columns I and IV, the percentage of glucose remained constant but in column IV, the peptone has been reduced to 0.5%. The following general conclusions can be drawn: (1) Anaerobes grown in medium with 0.5% peptone and 1% glucose produced considerable volumes of gas, which, however, are always smaller than those generated in 2% peptone solutions. (2) The CO_2/H_2 ratio is strikingly lower. The rise in the percentage of H_2 indicates two things: (a) the glucose is the source of the hydrogen; (b) the concentrated peptone solution promotes vigorous growth and favors side reactions, which utilize the H_2 resulting from the glucose decomposition. (3) The addition of 0.25% disodium phosphate to the medium (a) greatly increases the volume of gas, with a lower final P_{H} change; (b) generally lowers the CO_2/H_2 ratio producing an effect similar to that obtained by prolonged incubation. The increase in the relative amount of H_2 indicates that this gas is either evolved in greater amount as the fermentation progresses or that in the first part of the process side reactions utilize a greater amount of the hydrogen. (4) The addition of 1% sodium nitrate to the medium has the following effect: (a) It decidedly lowers the gas volume of *B. welchii* and *Vibrio septique*. (b) The CO_2/H_2 ratios of the proteolytic organisms are lowered. (c) The ratio in the gases of *B. welchii* and *Vibrio septique* is increased as much as 90 times; this reaction is apparently due to a reduction of the nitrate and a utilization of the liberated oxygen by the nascent hydrogen derived from the glucose.

Effect of Serum on Gaseous Fermentation.—Serum is often used to enrich mediums and to hasten growth. Although this further complicates the composition of the medium, a general idea of the change in gas evolution proved interesting. The sheep serum used was sterilized by passing it through a Berkefeld candle. It was added aseptically in 60 c.c. amounts to 240 c.c. of plain peptone solution. The final concentrations were 20% serum in 2% peptone water. Only *B. botulinus* and *B. sporogenes* were tested. Fundamental changes in the constituent gases were recorded.

Table 10A shows the data dealing with this experiment. It is pointed out by comparing the figures of this table with those of table 3A that the volume is increased and the reaction is more acid. The CO_2/H_2 ratio, with the exception of *B. botulinus* 53, is much lower, in fact,

nearly as low as in true glucose fermentation (table 5A). Furthermore, it is interesting to note the striking parallelism in the reactions produced by *B. botulinus* 38 and 26. Consistently, they give the highest CO_2/H_2 ratios typical for *B. botulinus*. Strain 38 is a type A, while the other strain is a type B. It has been stated before that no significant difference in gas analysis can be established between the two types.

The effect of 20% sheep serum on the glucose-peptone medium has also been investigated, and the data are recorded in table

TABLE 10
(A.) SERUM-PEPTONE FERMENTATION AFTER 7 DAYS' INCUBATION

Organism	Strain	Appearance of Medium	P _H Change	Volume at Standard, Cc.	CO ₂ %	H ₂ %	N ₂ %	Sum, %	Ratio CO ₂ /H ₂
1. <i>B. botulinus</i>	97	Black	7.0-6.5	113	81.2	14.8	3.8	99.8	5.5
2. <i>B. botulinus</i>	19	Black	7.0-6.6	140	83.5	14.0	2.4	99.9	6.0
3. <i>B. botulinus</i>	62	Black	7.0-6.4	185	86.0	3.0	10.9	99.9	2.9
4. <i>B. botulinus</i>	34	Black	7.0-6.6	208	87.5	5.2	7.1	99.8	16.8
5. <i>B. botulinus</i>	53	Black	7.0-6.6	105	87.1	8.6	4.3	100.0	10.1
6. <i>B. botulinus</i>	38	Black	7.0-6.4	210	92.4	4.9	2.4	99.7	18.8
7. <i>B. botulinus</i>	26	Black	7.0-6.6	245	91.7	5.8	2.2	99.7	15.8
8. <i>B. sporogenes</i>	48	Black	7.0-6.4	289	87.6	6.0	6.3	99.9	14.6
9. <i>B. sporogenes</i>	2	Black	7.0-6.4	200	86.4	8.5	5.0	99.9	10.2

(B.) GLUCOSE SERUM FERMENTATION AFTER 3 DAYS' INCUBATION

1. <i>B. botulinus</i>	97	Black	7.0-6.0	410	74.9	23.3	1.6	99.8	3.2
2. <i>B. botulinus</i>	19	Black	7.0-5.6	490	72.8	25.9	1.2	99.9	2.8
3. <i>B. botulinus</i>	62	Black	7.0-6.1	590	87.9	10.4	1.6	99.9	8.4
4. <i>B. botulinus</i>	34	Black	7.0-5.6	430	76.9	21.0	2.0	99.9	3.7
5. <i>B. botulinus</i>	53	Black	7.0-6.0	620	85.6	13.0	1.3	99.9	6.6
6. <i>B. botulinus</i>	38	Brown	7.0-5.7	510	64.7	34.2	1.0	99.9	1.9
7. <i>B. botulinus</i>	26	Yellowish	7.0-5.6	470	62.9	34.9	2.0	99.8	1.8
8. <i>B. sporogenes</i>	48	Black	7.0-5.6	535	74.6	22.8	2.4	99.8	3.3
9. <i>B. sporogenes</i>	2	Black	7.0-5.6	490	73.1	25.0	1.8	99.9	2.9

10B; the same incubation period of 3 days has been used. The serum greatly increases the volume of gas without altering the P_H. The CO_2/H_2 ratios for *B. botulinus* 62 and 53 and *B. sporogenes* 48 are slightly higher than those in table 5A. The ratios of *B. botulinus* strains 26 and 38 in this instance instead of giving the highest ratios as elsewhere, both give the lowest ratios. They parallel each other in the amount of the decrease. It is not unlikely that there is a high ratio group of *B. botulinus* strains which have certain metabolic activities in common. One recalls in this connection the studies of Rogers, Clark, and Davis,¹⁹ who found certain strains of *B. coli* to give consistently

¹⁹ Jour. Infect. Dis., 1914, 14, p. 411.

high gas ratios. In fact, they established definitely a high and low gas ratio group of these organisms. Their work was done with similar methods.

The general results of adding 20% sheep serum to a basic 2% peptone medium are: (1) The volume of gas is increased. (2) The serum acts as a protein buffer, and although growth is active and large amounts of gas are evolved, the P_H is not as low as otherwise occurs. (3) The evolution of H_2 is increased in proportion to the CO_2 , which results in a lower CO_2/H_2 than in the identical medium without the serum.

Effect of Decreasing the Volume of Medium.—In a series of experiments, the standard 300 c.c. volume of glucose-peptone medium in the 500 c.c. Kjeldahl culture flask was reduced to 150 c.c. The vacuum space over the medium was almost doubled, and the period of high vacuum during growth was lengthened. An unexpectedly large increase in the volume of gas was obtained per 100 c.c. of medium. The final P_H was slightly more acid with *B. welchii* and markedly lower with the proteolytic types. The latter also produced gases much richer in hydrogen, as evidenced by a decided lowering of the CO_2/H_2 ratios.

The greater gas volumes obtained throughout this experiment (table 11) indicate that the metabolic processes are more active at a lower vacuum pressure. This observation can be explained by the fact that the gas evolved into the larger vacuum space exerts about half the former pressure on the growing culture. Doubtless the accumulation of gaseous decomposition products exerts a depressing influence on the growth activities. The "vacuum buffer" has altered more decidedly both the volume and composition of the gas of the proteolytic organisms than either the addition of 0.25% Na_2HPO_4 or a 7-day incubation period. This statement can be verified by reference to previous tables. In order to compare the gas volumes, the following calculations have been made: The volume evolved from 150 c.c. of medium has been doubled, and the volume evolved from 300 c.c. of medium under former physical conditions has been subtracted from the previous figure. The volumes in the experiment under discussion are from 120 c.c. to 200 c.c. greater than those obtained from a week's incubation of the same medium (table 5B); they are from 200 to 300 c.c. larger than that evolved by adding 0.25% Na_2HPO_4 (table 8B) to the medium incubated for the same period. In view of this marked stimulation of gas production, it appears that the maintenance of the

conditions for optimum anaerobic growth demands the removal of accumulated catabolic gases. The culture tube described by Eldredge and Rogers²⁰ removes the main part of the gas (CO_2) by absorption in $\text{Ba}(\text{OH})_2$. The success which numerous workers have reported with this tube when dealing with fastidious aerobes, e. g., streptococci, is probably due to the favorable conditions for growth.

The gas constituents of *B. welchii* cultures are not altered; the CO_2/H_2 ratios duplicate those of the 300 c c. medium volumes. How-

TABLE 11
CHANGE IN THE GASEOUS METABOLISM DUE TO A DECREASED VOLUME OF MEDIUM

Ex- peri- ment	Organism	Strain	Medium Volume, Cc.	P_{H} Change	Gas Volume at Stan- dard, Cc.	CO_2 %	H_2 %	N_2 %	Sum, %	Ratio $\frac{\text{CO}_2}{\text{H}_2}$
1	<i>B. botulinus</i>	97	300	7.4-5.7	320	77.1	21.3	1.5	99.9	3.66
	<i>B. botulinus</i>	97	150	7.4-5.4	350	69.2	27.5	3.3	100.0	2.5
2	<i>B. botulinus</i>	53	300	7.4-6.0	435	76.9	19.9	3.1	99.9	3.86
	<i>B. botulinus</i>	53	150	7.4-5.2	375	69.1	28.9	1.8	99.8	2.4
3	<i>B. sporogenes</i>	46	300	7.4-6.4	305	84.0	13.1	2.8	99.9	6.4
	<i>B. sporogenes</i>	46	150	7.4-5.3	312	77.2	21.2	1.5	99.9	3.6
4	<i>B. sporogenes</i>	6	300	7.4-5.7	360	87.3	10.6	2.2	100.1	8.2
	<i>B. sporogenes</i>	6	150	7.4-5.4	210	76.5	20.5	2.8	99.8	3.7
5	<i>B. welchii</i>	26	300	7.4-4.6	310	50.3	47.8	1.7	99.8	1.05
	<i>B. welchii</i>	26	150	7.4-4.0	190	50.0	48.0	1.7	99.7	1.04
6	<i>B. welchii</i>	2	300	7.4-5.0	410	49.1	49.0	1.5	99.6	0.99
	<i>B. welchii</i>	2	150	7.4-5.0	265	48.2	49.6	2.1	99.9	0.97
7	<i>B. welchii</i>	129	300	7.4-5.0	195	45.2	53.1	1.7	100.0	0.85
	<i>B. welchii</i>	129	150	7.4-4.6	150	45.3	52.5	2.1	99.9	0.86
8	<i>B. welchii</i>	36	300	7.4-4.2	330	48.7	48.5	2.6	99.8	1.00
	<i>B. welchii</i>	36	150	7.4-4.2	200	48.0	48.3	3.4	99.7	0.99
9	<i>B. welchii</i>	135	300	7.4-4.2	320	48.8	49.0	2.0	99.8	0.99
	<i>B. welchii</i>	135	150	7.4-4.0	185	48.3	48.5	3.0	99.8	0.99

A glucose (1%) peptone (2%) medium was incubated for 3 days to obtain the above data.

ever, a decided lowering in the ratios from proteolytic cultures is noted. The ratios obtained from *B. botulinus* and *B. sporogenes* cultures are lower than those secured during a week's incubation in the presence of buffers. This increase in hydrogen may be due to the following factors: (a) Little of the evolved hydrogen is absorbed by side reactions, which are probably influenced by physical pressure. (b) The increased "vacuum buffer" favors growth and gas production, which cause progressive fractional metabolism in the glucose peptone medium.

²⁰ Centralbl. f. Bakteriöl., 11, 1914, 40, p. 5.

SUMMARY

The results of the investigation of the gaseous metabolism of 25 strains of anaerobic bacteria are presented in detail. A basic medium consisting of "Difco" peptone (2%), modified by the addition of various salts and carbohydrates, has been used in order to determine the effect on the metabolic processes and the progressive stages of decomposition. The results secured in this manner have been duplicated with the same cultures several months after the original analyses had been made. It must, however, be emphasized that the established standardized technic must be strictly adhered to in order to secure comparable results. Changes, either in the formula of the nutrient broth or in the physical environment of the growing culture, alter the volume and the composition of the gaseous end products appreciably.

The gases evolved in the course of growth by anaerobic bacteria are CO_2 , H_2 and small amounts of N_2 . No evidence of the production of NH_3 , N_2O , CO , CH_4 or other carbonaceous gas has been found. The liberation of 0.2% to 0.4% oxides of nitrogen other than N_2O is considered as too small an amount to warrant their determination. The odorous gas fraction is composed mostly of H_2S but doubtless, volatile amines, thioethers and mercaptans contribute to the foul smell. Lack of satisfactory methods prevented the direct estimation of the H_2S gas. However, determinations of the sulphur metabolized make it probable that at least 2% to 3% H_2S are formed by proteolytic bacteria.

Changes in the constituent gases are traced by means of the quotient ratio from the fraction $\frac{\text{percentage } \text{CO}_2}{\text{percentage } \text{H}_2}$ shown in the analyses. A high percentage of hydrogen causes the ratio to approach unity, which figure indicates that a saccharolytic metabolic process has been in progress. The following general conclusions (from tables 4, 6, 9 and 10) can be drawn regarding the reactions of the various species in different mediums:

(1) *B. botulinus*, a proteolytic anaerobe, is capable of saccharolytic activities in the presence of scant nitrogenous material. In plain peptone solution, during a 7-day incubation period, the CO_2/H_2 ratio for 7 strains averages 18.3. The incorporation of 20% sheep serum increases the gas volume and decreases the ratio to 10.9. The average 18.3 is lowered to 5.7 when sodium formate is added to the peptone. The reduction (6.1) is not as marked in the presence of sodium chloride. Salicin is added to the peptone, which furnishes an average gas ratio of 5.0. At the

end of a 3-day glucose fermentation, the quotient averages 6.2, but on prolonged incubation (7 days) it is 3.8. The addition of several salts to a 3-day glucose-peptone fermentation decreases the average ratio (6.2) as follows: NaNO_3 to 3.5; Na_2HPO_4 to 3.2; HCOONa to 3.2, and NaCl to 2.7. Incorporation of 20% serum in the glucose peptone fluid stimulates growth and increases the gas ratio to 4. Vigorous growth increases not only the volume but also the H_2 . The gas ratio is decreased to 1.9, indicative of a saccharolytic process, when the peptone concentration is lowered to 0.5% in a 1% glucose medium. No analytic differences in the gas composition between *B. botulinus* types A and B have been noted.

It is observed that there are strains (e. g., Nos. 40, 38, 34 and 26) which evolve gas relatively poor in insoluble H_2 (1% to 3%), and constitute a group with high CO_2/H_2 ratios. This observation may explain the lack of bulging tins with food products containing potent *B. botulinus* toxin. Epidemiologic investigation of human botulism outbreaks has shown that toxin production is not always coincident with visible signs of spoilage, especially production of gas. All these strains produce H_2 more vigorously than strain 40; the latter was isolated from a human outbreak due to apparently sound commercially canned beets. The heat resistant, spore-bearing anaerobe, *B. sporogenes*, which frequently survives heating gives a higher average CO_2 output in general than *B. botulinus*. Consequently, food contaminations due to this species might pass unnoticed unless the proteolysis had disintegrated the fibers of the product.

(2) The data indicate that *B. sporogenes* is more highly proteolytic than *B. botulinus*. The average CO_2/H_2 ratios in 2% peptone at the end of 7-day incubation are 36.9 and 18.3, respectively. Modifications of the plain peptone fermentation decreases the ratio of 36.9 as follows: 20% sheep serum to 12.4; HCOONa to 5.7; NaCl to 14.1. It is noted that the chloride does not stimulate as greatly as the formate. Salicin added to the peptone furnishes an average gas ratio of 29.9. Although the volumes of gas are larger, this ratio is slightly lower than that obtained in plain peptone, which indicates more active growth. However, *B. sporogenes* does not ferment this glucoside with the same vigor with which glucose is decomposed. This species is classified as a nonsalicin fermenter because little H_2 is generated from this carbohydrate. The ratio, after 3 days' growth in glucose, is 5.6 (cf. to 29.9 in salicin). The average ratio in glucose is decreased to 4.7 by prolonged incubation (7 days).

The addition of several salts to a 3-day glucose peptone fermentation decreases the average ratio (5.6) as indicated: NaNO_3 to 4.7; Na_2HPO_4 to 2.9; HCOONa gives 5.6, and NaCl to 4.8. The incorporation of 20% serum to the glucose-peptone fluid lowers the ratio to 3.1 and increases the total gas volume. In the same glucose-peptone medium, when the peptone is decreased from 2% to 0.5%, the ratio is 1.9. The same ratio is encountered when *B. botulinus* is grown in the scant nitrogenous fluid; it is evident that both species are capable of saccharolytic metabolism when the main source of energy is glucose. Furthermore, these observations show that the sugar is the main source of H_2 , and that the evolution of H_2 causes positive results usually in qualitative tests.

There is no CO_2/H_2 ratio characteristic of *B. botulinus* or *B. sporogenes*. However, the gas ratios from salicin peptone mediums differentiate between them as a rule. *B. sporogenes* liberates little H_2 from salicin, and this fact is recorded in the high quotient ratios.

(3) *B. histolyticus* gives only traces of H_2 on all 2% peptone mediums. The gas ratio on a 60 c.c. sample from plain peptone culture was 91 +, indicating an extreme degree of proteolysis. In a glucose-peptone medium, no H_2 was formed until the peptone was reduced to 0.5%. The latter experiment yielded a gas ratio of 11.6 from a 20 c.c. gas sample. Gas analyses indicate that the organism can decompose glucose slightly in a nitrogen-poor broth.

(4) The gaseous metabolism of *B. centrosporogenes* 82 on peptone medium without sugar resembles that of *B. sporogenes*. The gas ratio on plain peptone is 37.4; when formate and chloride salts are added, this figure drops to 25.5 in both instances. However, the reaction in the presence of glucose has a greater saccharolytic tendency than with the latter organism. Three-day incubation in the presence of glucose gives a ratio of 2.1, and this number is decreased to 1.7 during a 7-day period. There is no appreciable change in the gas ratio (2.1) as the buffer increases the gas volume from a glucose-peptone medium. Nitrate depresses the evolution of gas and increases the ratio to 3.3; glucose added to a 0.5% peptone solution yields considerable gas and a CO_2/H_2 ratio of 1.5.

(5) Two strains of *B. tetani*, grown in a glucose peptone solution, evolved 50 c.c. of gas after 7 days. This species is generally regarded as a nongas-producer when the customary qualitative technic is employed. The small volume of gas extracted by vacuum gave a

CO_2/H_2 ratio of 1.17. Apparently the feeble catabolic process during the first week of growth is essentially saccharolytic, although the black appearance of the medium indicates some proteolysis.

(6) The gases liberated from the cultures of *B. welchii* are rich in H_2 (50% or more). Although this statement is true in plain peptone solution when the ratio is 0.4, the volumes of gas evolved without sugar are very small. The addition of NaCOOH and NaCl does not increase the gas volumes, but decreases the ratios to 0.19 and 0.21, respectively. However, 3 days' propagation in the presence of glucose yields volumes from 410 c.c. to 240 c.c., with an average gas ratio of 1. A week's incubation with excess carbohydrate present leads to a ratio of 0.98. Salicin is fermented by 3 of the 6 strains which liberate appreciable volumes of gas with a ratio composition around unity. The 3 nonfermenting strains give the same gas volume as in peptone, but the average salicin ratio is lowered to 0.55. The addition of phosphate reduces the gas ratio to 0.85. Sodium nitrate (1%) added to a glucose-peptone medium creates a striking change in the gas composition. The total volumes are decreased more than with proteolytic organisms. However, *B. welchii* reduces the nitrate so vigorously that nearly all of the nascent H_2 is oxidized by oxygen from the nitrate. Consequently, the CO_2/H_2 ratio is increased from unity to 52.1. On the other hand, nitrate in cultures of proteolytic bacteria decreases instead of increases the gas ratio. The nitrite radical is also reduced by *B. welchii* (ratio 7.7) but depresses gas production markedly. The ratio of gases is decreased from 1 to 0.67 in a 1% glucose medium when the peptone concentration is lowered to 0.5%. This observation shows, even in this relatively pure carbohydrate medium, that the glucose is not decomposed into equal volumes of CO_2 and H_2 , as is generally quoted in the literature.

(7) Gas analyses on cultures of 2 strains of *Vibrio septique* indicate that this organism closely resembles *B. welchii*. It is classified as a purely saccharolytic micro-organism. The gas volume evolved from plain peptone is low, but the CO_2/H_2 ratio is 0.98. A 3-day glucose fermentation yields less gas than *B. welchii* cultures, but the average ratio is 0.99. *Vibrio septique* resembles *B. welchii* in the matter of nitrate reduction in the presence of glucose; however, the reduction is not so vigorous. The gas ratio with this species is increased from unity to 5.3 (*B. welchii* ratio 52.1). The oxidation of nascent H_2 by oxygen derived from reduction of the nitrate radical is character-

istic of these two highly saccharolytic species. One strain of *Vibrio septique* is a salicin fermenter; the ratio of this strain is 0.96 from a 175 c.c. sample.

(8) *B. bifermentans* is feebly proteolytic. A single strain grown for 7 days in plain peptone evolved 46 c.c. of gas with a ratio of 45.1; the magnitude of the latter figure is indicative of proteolysis. The ratio is decreased to 8.7 and 11.4 by the addition of HCOONa and NaCl , respectively. The gas ratio in a glucose-peptone medium (1.66) resembles a process similar to that of *B. welchii*. However, *B. bifermentans* does not resemble the other two saccharolytic organisms in its reduction of nitrates. The gas ratio in nitrate glucose medium is decreased to 1.05; in this respect, the organism resembles proteolytic bacteria. Less H_2 is formed from salicin than from glucose, as shown by the ratio of 18.6. The strain can be classified as salicin negative.

CONCLUSIONS

The gaseous metabolism of *B. botulinus*, *B. sporogenes*, *B. centrosporogenes*, *B. welchii*, *B. bifermentans*, *B. tetani*, and *Vibrio septique* has been studied by a standardized technic, and the results are presented in detail.

The main gases evolved in the course of anaerobic cultivation are CO_2 , H_2 and small amounts of N . The fractions of the oxides of nitrogen, mercaptans, thioethers and amines are too small to warrant their determination. The odorous gas is composed mainly of H_2S which constitutes 2%-3% of the total gas volume.

Changes in the constituent gases are traced by means of the quotient ratio from the fraction $\frac{\text{percentage } \text{CO}_2}{\text{percentage } \text{H}_2}$ shown in the analyses. A high percentage of hydrogen causes the ratio to approach unity, indicating that a saccharolytic process has been in progress. Anaerobic organisms can be readily classified according to their gaseous metabolism.

FORMATION OF ACROLEIN FROM GLYCEROL BY *B. WELCHII*

FREDERICK B. HUMPHREYS

*From Bacteriological Laboratory of Presbyterian Hospital, and Department of Bacteriology,
College of Physicians and Surgeons, Columbia University, New York City*

The action of *B. welchii* on glycerol has been studied by various observers, chiefly with the object of subclassifying the group according to whether or not they ferment this substance. Simonds¹ investigated 20 strains with the following results:

Fermenting both inulin and glycerol, 20%; fermenting glycerol but not inulin, 35%; fermenting inulin but not glycerol, 25%; fermenting neither inulin nor glycerol, 20%.

Henry² in general confirms Simonds results, although his 20 strains were distributed differently among the subgroups:

Fermenting both inulin and glycerol, 45%; fermenting glycerol but not inulin, 20%; fermenting inulin but not glycerol, 25%; fermenting neither inulin nor glycerol, 10%.

Kendall, Day and Walker³ investigated the action of 12 strains of *B. welchii* on glycerol, and found that 6 gave a strong increase of titratable acidity, 3 a slight increase, and 3 none. They believe that this glycerol fermentation is an exception to the rule that *B. welchii* can ordinarily attack only the aldehyde group of the hexoses and disaccharides, but not the alcohol groups of mannitol, etc. They explain the fermentation of saccharose (which, as such, contains no aldehyde group) by assuming a preliminary splitting of that disaccharide into dextrose and fructose.

While attempting to subclassify our strains of *B. welchii*, isolated from a variety of sources, we noted the fact, which, we have not found recorded in the literature, that subplants made from 18-24 hour cultures of *B. welchii* in 2% glycerol broth to blood-agar plates and incubated anaerobically, uniformly fail to grow. These glycerol cultures themselves developed a diffuse turbidity; and stained preparations always

Received for publication, April 17, 1924.

¹ Jour. Infect. Dis., 1915, 16, p. 31.

² Jour. Path. & Bacteriol., 1916-17, 21, p. 372.

³ Jour. Infect. Dis., 1922, 30, p. 141.

showed abundant normal appearing gram-positive bacilli. These, however, were apparently dead and never proliferated when streaked on a plate.

This experiment has been repeated many times, using various brands of glycerol and scores of different strains of *B. welchii*, including several standard strains from American and European collections. We have never encountered any strain consistently exceptional in this regard. In retesting some of our old stock cultures which had been stored in meat broth on ice for 1 to 2 years, we sometimes found that the power to grow in glycerol broth or other mediums was markedly diminished. In these cases, the glycerol broth would remain practically clear during our usual period of 18-24 hour incubation, except for a slight sediment at the bottom of the tube. From these, viable transplants were usually obtained. Rejuvenation of such cultures by a series of rapid transferences in favorable mediums, seeding with large amounts, always restored to them their original power of growth in glycerol mediums; and from these rejuvenated glycerol-broth cultures, no viable subplants were ever obtained.

Evidently some bactericidal or growth inhibiting substance is formed by the bacteria in their growth in glycerol. That the death or inhibition is not simply due to an increase of acidity is demonstrated by the fact that 1% dextrose broth cultures, made at the same time and attaining a relatively high hydrogen-ion concentration during the 18-24 hour period of incubation, always give an abundant growth on transplant, whereas many of the nontransplantable glycerol broth cultures remain practically neutral.

The substance formed in glycerol is bactericidal, as is shown by the fact that when another organism, such as *B. proteus* is sown in the glycerol broth with the *B. welchii*, neither organism after incubation and growth in the glycerol grows on transplant; whereas control cultures of *B. proteus* alone in glycerol broth remain entirely viable, as do also the much more acid dextrose broth cultures of the 2 organisms combined. The same results may be obtained with combinations of *B. welchii* and a variety of other organisms, including *B. pyocyaneus*, *B. coli*, *Vibrio septique*, and *BB. oedematiens*. The last organism by itself forms considerable acid and gas from glycerol, and when grown alone remains perfectly viable therein; but when grown with *B. welchii*, is promptly killed.

The centrifugalized supernatant fluid of our glycerol-broth cultures of different strains of *B. welchii* varies greatly in acidity. Indeed, we

obtained all grades of acidity from 0 to + + +, (P_H 7 to P_H 4.5), using the indicators and scale suggested by the committee of Assoc. of Am. Bacteriologists.⁴ Thus, of 50 strains:

Thirty-six per cent. gave 0 acidity (P_H 6.2 or more); 8% gave — + acidity (P_H 6.1); 12% gave + acidity (P_H 5.2—6); 8% gave — + + acidity (P_H 5.1); 32% gave + + acidity (P_H 4.6—5); 4% gave + + + acidity (P_H 4.6 or less).

Sometimes the fluids had a slight peculiar reddish-orange color and usually, especially on gentle warming, gave off an acrid odor suggestive of acrolein. Acting on this suggestion, we tested our supernatants for this substance, (a) by Schiff's aldehyde reagent, and (b) by Voisenet's "proteic acid test" or so-called "glycero-reaction."⁵ All gave positive reactions for acrolein.

The technical methods used may be summarized as follows:

All the strains of *B. welchii* were of characteristic morphology and showed typical sugar fermentations and other usual cultural reactions, including "stormy fermentation" of milk. Scrupulous attention was given to obtaining and maintaining purity of our cultures by constant and repeated plating and fishing of well isolated colonies from the surface of blood-agar plates.

For anaerobiosis we depended entirely on a slightly modified McIntosh and Fildes palladium jar, with the usual control tube of methylene blue dextrose broth.

For fermentation reactions, ordinary plain beef infusion broth was the basis. Previous fermentation by yeast, colon bacilli, etc., to free it of muscle sugar, was found to be superfluous; as it was found that when *B. welchii* was grown in this simple broth without the addition of sugar, the culture never became sufficiently acid to cause confusion, i. e., always remained green or blue to brom thymol blue. The sugars used (C. P. dextrose, lactose, saccharose, and salicin) were added in 1% amounts and the medium sterilized in the autoclave at 10 pounds for 15 minutes. We do not consider "fractional sterilization" safe for anaerobic work; and it has been shown⁶ that there is more change in sugar mediums after prolonged and repeated exposure to a temperature of 98-100 C. than after a single short one to a relatively higher temperature. In the case of glycerol, 2% by volume was used. Various brands were tried, including the "U. S. P. Pure," Powers-Weightman-Rosengarten "Special Analytical," and the "Special Absolutely Pure" of the National Aniline and Chemical Company. The results were identical in all cases. No noticeable difference in results was obtained whether the medium was heated in the autoclave at 10 pounds for 15 minutes after the addition of the glycerol, whether it was fractionally sterilized in the Arnold, or whether the glycerol was added to the sterilized base medium directly from a freshly opened bottle of pure glycerol, the latter thus being subjected to no heat. Autoclave sterilization was therefore employed here also. No litmus or other indicator was added. The final P_H of the medium was regularly about 7.4.

The fermentations were usually carried out in tubes of the ordinary "fish-hook" pattern. The tubes were heated in the water bath for 10 minutes to expel the dissolved air, cooled rapidly, and immediately seeded. The inoculums gen-

⁴ Jour. Bacteriol., 1919, 5, p. 136.

⁵ Voisenet, E.: Ann. de l'Inst. Pasteur, 1918, 32, p. 476.

⁶ Mudge, C.: Jour. Bacteriol., 1917, 2, p. 403.

erally consisted of 2 drops of the fluid portion of an actively growing 18-hour meat-broth culture, or sometimes of a colony from a fresh blood-agar plate. The cultures were incubated about 18-24 hours in the palladium jar at 37 C. On removal, smears for staining and subcultures on blood-agar plates were made. They were then centrifugalized and the clear supernatant fluids tested for the degree of acidity, for aldehydes in general, and for acrolein in particular.

Acidities were roughly determined and recorded according to the schema proposed by the committee of Assoc. of Am. Bacteriologists.⁴

"Neutral," 0 P_H 6.2 or more, Blue or Green to Brom-thymol Blue. "Slight Acid," + P_H 5.2-6, Yellow to Brom-thymol Blue; Purple to Brom Cresol Purple. "Moderately Acid," ++ P_H 4.6-5, Yellow to Brom Cresol Purple; Orange to Methyl Red. "Strongly Acid," +++ P_H 4.5 or less, Full Red to Methyl Red.

Tests were made for aldehydes in general by means of Schiff's aldehyde reagent, made according to the formula of Leys, as quoted by Voisenet⁵ as follows:

0.1% aqueous solution basic fuchsin, 1000 cc.; sodium bisulphite, 30° Baume, (about 25%) 10 cc.; concentrated hydrochloric acid, 10 cc.

The bisulphite and fuchsin solutions are mixed. When the color has become markedly attenuated, the HCl is added. The liquid at first takes a brownish tint, but after standing a few days becomes colorless. With some brands of domestic fuchsin there always remains a golden yellow color, which apparently, however, in no way interferes with the test.

In carrying out the test, we have been accustomed to pipette 1 cc. into a small test-tube, and add ½ cc. of the clear supernatant fluid from the culture. If even a trace of aldehyde is present, a violet red color will develop in the course of a few minutes. Unless an excess of the reagent is used, false reactions will be obtained. We have found that equal parts of the reagent and of plain sterile broth will give a red coloration, especially when layered on without mixing. When, however, the proportion of 2:1 is employed, with immediate and thorough mixing, such false reaction do not occur.

Acrolein more specifically was tested for by means of Voisenet's "Glycero-reaction"⁵ as follows: Reagent A: (Nitro hydrochloric acid); pure concentrated hydrochloric acid (sp.gr. 1.18), 200 cc.; 3.6% aqueous potassium nitrite, 0.1 c.c. Reagent B: (Albumin water), the white of one egg is beaten up with 5-7 cc. of distilled water and filtered under pressure, resulting in about a 10% albumin solution.

To apply the test, mix 5 cc. of the test fluid, 1 cc. of the albumin water, and 18 cc. of the nitro hydrochloric acid. Shake to dissolve the precipitated egg albumin and put in a water bath of 50 C. In a few minutes, in the presence of a trace of acrolein, a green or greenish-blue color appears. This color depends on the interaction of the aldehyde, the nitrous acid, and the tryptophan formed by the action of the hydrochloric acid on the protein. Formaldehyde gives a violet color; acetaldehyde, purple; hydracrylic aldehyde, grayish-red, etc.

We have modified the test slightly, using smaller amounts of test fluid and substituting undiluted human serum ("leave-over" Wassermann serums) for the albumin water: Mix in a small 3 by 3/8 test-tube, clear supernatant fluid from centrifugalized culture, 0.25 cc.; undiluted serum, 0.25 cc.; nitrohydrochloric acid, 1.00 c.c. Immerse in the 55 C. water bath for 2 or 3 minutes and let stand at room temperature for 5 to 10 minutes.

The reaction is not a quantitative one. Indeed, strong acrolein solutions may give apparently negative reactions or a hardly noticeable light yellowish, dirty green. Therefore, if there is a positive Schiff test and a distinct odor suggestive of acrolein, and still no green or blue color given with Voisenet's test, the test

should be repeated using dilutions of the supernatant fluid with plain broth. The nitro hydrochloric acid deteriorates easily and should be freshly prepared and kept in a dark bottle.

Over 50 strains of *B. welchii* have been tested, with uniform results. The uniform acrolein production and the destruction thereby of the bacteria with varying acidity production as shown in 50 consecutive instances are summarized in table 1.

TABLE 1
ACID AND ACROLEIN PRODUCTION BY 50 STRAINS OF TYPICAL *B. WELCHII*

Strains	Acidity	Viability	Schiff's	Voisenet's
8, 18, 20, 24, 25, 42, 43, 45, 60, 65, 68, 86, 110, 111, 136, 145, 198, 240.....	0	Lost	+	+
51, 57, 88, 98.....	- +	Lost	+	+
4, 10, 11, 19, 94, 132, 144.....	+	Lost	+	+
64, 124, 167, 239.....	- + +	Lost	+	+
1, 9, 13, 16, 27, 44, 46, 58, 83, 109, 175, 184, 195, 217, 225	+ +	Lost	+	+
28, 125	+ + +	Lost	+	+

Of these 50 strains, derived from various sources, 64% hemolyzed sheep red blood cells, while 36% were nonhemolytic. Some were highly pathogenic for guinea-pigs on intramuscular injection, others much less so or not at all. We could discern no relationship between the hemolyzing power, the pathogenicity for guinea-pigs, the degree of acid production in glycerol medium, and the source of the strain.

Supernatant fluids from dextrose and plain broth cultures of *B. welchii*, when tested for acrolein in the manner described, are always negative. Supernatant fluids from glycerol-broth cultures of a considerable variety of organisms other than *B. welchii*, aerobic and anaerobic, likewise give uniformly negative results (table 2). We have encountered several strains of anaerobic organisms, differing somewhat in certain cultural characteristics from the typical *B. welchii*, but which in glycerol medium behave in a precisely similar manner. These, for immunologic reasons, we consider to be atypical *B. welchii* and will be made the subject of a separate article. None of these atypical forms are included in table 2.

We believe, therefore, that we are justified in making the following general statement:

All members of the *B. welchii* group, whether or not they are able to make acid and gas from glycerol, form the aldehyde acrolein from it.

And, among the more common anaerobes, at any rate, *B. welchii* is unique in this regard. This characteristic is, then, diagnostic for the group.

Acrolein or acrylic aldehyde is the aldehyde of the unsaturated acrylic acid. It is a colorless, volatile fluid (B.P. 52 C.) with an exceedingly pungent odor, irritating to the mucous membranes when at all concentrated. It is formed from glycerol by the abstraction of 2 molecules of water on heating with such agents as acid potassium sulphate. On the other hand, under various circumstances, it easily becomes polymerized to disacryl or metacrolein; and, on exposure to air may become oxidized to the corresponding acrylic acid. Its bactericidal and antiseptic properties have been investigated by Koch and Fuchs,⁷ who found that 0.5% of acrolein will kill *B. pyocyaneus*, *B. Coli*,

TABLE 2

ACTION OF MISCELLANEOUS ORGANISMS OTHER THAN *B. WELCHII*, IN 2% GLYCEROL-BROTH

Organisms	Acidity	Viability	Schiff's	Voisenet's
<i>B. typhosus</i> , <i>paratyphosus</i> A, <i>paratyphosus</i> B, <i>B. pestis-caviae</i> , <i>coli-communis</i> , <i>coli-communior</i> , <i>acidi-lactici</i> , <i>proteus-vulgaris</i> , <i>pyocyaneus</i> , <i>subtilis</i> , <i>sporogenes</i> , <i>histolyticus</i> , <i>botulinus</i> A, <i>botulinus</i> B, <i>tertilus</i> ; <i>tetani</i> , <i>Staphylococcus albus</i> , <i>Streptococcus pyogenes</i> , <i>mitis</i> , <i>Pneumococcus</i> , <i>Meningococcus</i> , <i>Vibrio septique</i>	0	Good	0	0
<i>B. dysenteriae</i> -Flexner, <i>diphtheriae</i> , <i>Staphylococcus aureus</i>	+	Good	0	0
<i>B. bifermentans</i>	- + +	Good	0	0
<i>B. oedematiens</i>	+ +	Good	0	0

Staphylococcus aureus and *albus* in 5 minutes, while 0.25% solutions require 1 hour.

The production of acrolein in any appreciable quantity from glycerol by bacterial action has been noted, so far as we can find, by but one observer, E. Voisenet,⁵ who described an aerobic organism of the colon bacillus group, which he isolated from water and from wine with "bitter disease," and which, under proper conditions, forms appreciable amounts of acrolein in glycerol medium. He has named it *B. amaracrylus* from its supposed etiologic relationship with this wine disease. Voisenet believes the acrolein-forming power of *B. amaracrylus* to be unique and discusses at length the conditions and significance of its production. We must refer to the original article for the details but call attention to the fact that he shows the formation of acrolein from glycerol to

⁷ Centralbl. f. Bakteriöl., 1899, 26, p. 560.

be an exothermic reaction and may therefore serve as a source of energy for the growing bacterium. He believes, moreover, that this organism not only forms acrolein but can consume it, i. e., that acrolein is an intermediate product in the bacterial metabolism of glycerol.

The latter suggestion is of peculiar interest in connection with the opinion of Kendall, Day and Walker³ that *B. welchii* can form acid and gas only from substances possessing an available aldehyde radicle. If acrolein is looked on as an intermediate product in *B. welchii* metabolism of glycerol, the ability of the organism to form acid and gas from this substance, providing it is not first killed by too great an accumulation of the aldehyde, is explained. If the intermediate product, i. e., the bactericidal aldehyde acrolein should accumulate sufficiently so as to reach rapidly a concentration that is inimical to the activities of the organism, we could hardly expect as much acid and gas to be formed as when this lethal concentration is more slowly attained. The final acidity attained, therefore, would depend on the relative speeds of the formation and of the consumption of the acrolein; and hence, as these speeds must vary under many conditions quite beyond our exact control, we would expect all grades of acidity to be found in the various cultures when the lethal point is finally reached. And such, in fact, is the case.

We recognize, of course, that individual differences may exist in the susceptibility of various strains of *B. welchii* to acrolein. But such differences are of minor biologic importance, and could scarcely be considered enough to warrant the grouping suggested by Simonds.¹

These considerations vitiate, in our opinion, the use of the "fermentation" of glycerol as a basis for the subclassification of the group.

Simonds¹ does not give his exact technic in determining his acidities or of obtaining his anaerobiosis, but simply makes the statement that small amounts of gas did not mean fermentation and that "if there was no increase of acidity, especially if spores were present, it was believed that the strain had not acted on the carbohydrate." A close analysis of his work is, therefore, impossible.

Henry,² on the other hand, gives his exact technic. He used a casein digest medium containing a Durham tube and 1% of the fermentable substance under investigation, covered with liquid paraffin and sterilized for 1½ hours. Just before inoculation, a few drops of his alkaline egg medium was added as an indicator. His inoculum was a small amount of the fluid portion of a vigorously growing meat-broth culture. He incubated for 48 hours before reading, although the reaction was usually complete in 15-24 hours. Positive fermentation (acid formation) was

indicated by the precipitation of the egg albumin. Sometimes when there was doubt about the precipitation, he used litmus. He says: "We are inclined to believe that freshly isolated *B. welchii* from wounds ferment both substances (inulin and glycerol); that one or the other reaction is first lost; then both disappear after prolonged sojourn on artificial media."

It seems somewhat arbitrary to select some particular acidity, the attainment of which is taken as indicating "fermentation," and the non-attainment of which, the nonutilization of the carbohydrate under consideration. By experimenting with Walpole's acetic acid sodium acetate mixtures, we found that in the mixture P_H 5.2 an immediate heavy precipitate formed on the addition of a few drops of alkaline egg medium made according to the formula given by Henry. In the solution P_H 5.4, the precipitate formed only slowly after standing some time; while at P_H 5.6 the fluid remained clear indefinitely. If, then we take P_H 5.2 as the acidity corresponding to Henry's frank positive fermentation, and P_H 5.4 as his doubtful ones, cultures attaining an acidity of P_H 5.6-6.0 would be, for him, definitely negative. Yet such cultures really would be distinctly, if slightly, acid; i. e., yellow to brom-thymol blue or + according to the scale used by us. A considerable number of our glycerol cultures showed this slight increase of hydrogen-ion concentration.

Although usually the final acidity of a culture growing in a medium containing an abundance of a fermentable carbohydrate may correctly be assumed to be dependent on the degree of acidity necessary to inhibit growth, the fact must not be lost sight of that growth-inhibiting substances other than acid may be formed during the process of fermentation.

We believe that we have shown that such a growth-inhibiting substance—acrolein—is regularly formed in glycerol medium by all strains of *B. welchii*; and that, therefore, the final acidity of the culture is not a safe guide for determining the inherent ability of the organism to utilize glycerol.

SUMMARY

In our experience, all strains of *B. welchii* are able to form the aldehyde acrolein in glycerol broth in sufficient quantity after 18-24 hours' incubation to inhibit growth and kill the organism.

The formation of acrolein from glycerol is an important differential characteristic of *B. welchii* and is believed by us to be diagnostic for the *B. welchii* group.

The final acidity of these cultures varies greatly, and cannot properly be taken as a measure of the ability inherent in the organism to utilize glycerol. Therefore, subclassification of *B. welchii* group on the basis of glycerol fermentation, determined by the acidity of the culture to this or that indicator, is unsound biologically and serves no useful purpose.

By assuming that acrolein is an intermediate product in *B. welchii* metabolism of glycerol, an explanation is offered for the exception in the case of glycerol, to the hypothesis suggested by Kendall, Day and Walker that this micro-organism cannot directly ferment carbohydrates which do not contain an aldehyde radicle.

B. PESTIS IN BLOOD, BILE AND URINE

OSAMU OHOTO

From the Momoyama-Hospital for Infectious Diseases and II. Medical Clinic, Medical College, Osaka, Japan

Although numerous investigators have examined the blood of patients with plague for *B. pestis*, no one has yet advanced any convincing explanation of the relation of bacilli in the blood to the development of plague.

From the results of examination of test animals, Tsurumi and his coworkers¹ and others state that in plague infection the circulating blood acts only as a carrier of bacilli from the infected location to other tissues, but that the appearance of bacilli in blood is not a specific condition for the development of the disease. According to Sata,² who studied this subject in detail both bacteriologically and anatomically, at the beginning of the infection, bacilli are held chiefly at the point in which inoculation took place, or in neighboring lymph nodes. The bacilli, however, have a great tendency in time to enter the circulating blood and grow in it. As a result, there occurs an intensive production of toxin with the characteristic severe symptoms and plague septicemia develops.

As to the presence of bacilli in the bile, no detailed examination appears to have been made. In Tsurumi's experiments in 13 fatal human cases of plague and 9 animal cases, no positive result was obtained, and he ascribed this to the unfavorable conditions in bile for the growth of pest bacilli.

In a previous paper³ on *B. pestis* in blood of patients with plague, it is pointed out that the examination for *B. pestis* in the circulating blood is best made by cultivating on bile medium or 1% sodium citrate broth. By this method, 72.2% of patients with pest gave positive results. Consequently, pest septicemia is not rare as reported heretofore, and its prognosis not absolutely bad. The presence of *B. pestis* in the blood should not be explained as due only to the invasion of the blood after it has been growing at the point of invasion; it may grow at the same

Received for publication, April 23, 1924.

¹ Nisshin Igaku, 1922, 12, p. 447.

² Arch. f. Hyg., 1900, 37, p. 105.

³ Ohoto, O.: Japan Med. World, 1923, 3, p. 136.

time in the blood and organs after its entrance in the body. Since then further examination of animals as to the presence of bacilli in the blood, bile and urine has been made, and results are reported here.

Culture Mediums.—For growing the bacilli both citrated broth and bile mediums, prepared in the following manner, were used chiefly, because, as stated in the first report, they present favorable conditions for growth of the organisms.

Citrated Broth: Liebig's beef extract, 5 gm., 5 gm. of pure peptone, and 5 gm. of sodium citrate are dissolved in 500 c.c. of distilled water. The solution is heated for 2 hours in Koch's steam sterilizer and the reaction adjusted to neutral or slightly alkaline. Of such solution, 10 c.c. is placed in each tube and sterilized once more for one hour.

Bile Medium: After addition of 5 gm. of pure peptone, 500 c.c. of fresh ox bile are sterilized for 2 hours in steam sterilizer and divided equally into 50 test tubes and then heated again for one hour.

Stock Culture: The strain, used in each experiment, was isolated last year from the blood of a plague patient. After the original planting on bile and agar mediums, it had been allowed to stand at room temperature for 4 months. In spite of this, the strain held its virulence without remarkable decrease and 1/100 of a loop was enough to kill a guinea-pig of 240 gm. within 98 hours on cutaneous inoculation.

Test Animals and Inoculation: Nine guinea-pigs, weight from 200-300 gm., were divided into 3 groups, the first group was inoculated cutaneously, the second subcutaneously, and the third intraperitoneally with definite amounts of the stock culture

APPEARANCE OF BACILLI IN CIRCULATING BLOOD

After inoculating 1/1,000-1/10,000 of a loop of the stock culture in each group of guinea-pigs, 0.6 c.c. of heart blood was obtained from each at various intervals and 0.2 c.c. placed in tubes of each of the 3 culture mediums, bile, citrated broth and slanted agar, and incubated for about 48 hours at 30 C., when the results of growth were determined (table 1).

TABLE 1
BACILLI IN BLOOD

No. of Animal	Weight in Grams	Inoculation and Amount	Lapse of Time After Inoculation											
			10 Min.	30 Min.	1 Hr.	2 Hr.	3 Hr.	5 Hr.	8 Hr.	12 Hr.	24 Hr.	48 Hr.	72 Hr.	96 Hr.
16	230	Cutaneous, 1/1,000 of loop	0	0	0	0	0	+
17	235		...	0	0	0	+	+
18	245		0	0	0	+
19	255	Subcutaneous, 1/1,000 of loop	0	0	0	+	+	+
20	255		...	0	0	0	...	+	+	+
21	270		0	0†
22	270	Intraperitoneal, 1/10,000 of loop	+	0	0	+	+	+
23	290		...	0	0	0	+	+
24	300		0	0	+	+	+	+

* Died. † Died after heart puncture.

While in my experiment on patients with plague the presence of bacilli in the circulating blood was demonstrated in 72.2% of 36 cases, on artificial infection every blood sample obtained just before the death of the animal gave positive results, after cutaneous inoculation within 72 hours, after subcutaneous within 48 hours and after intraperitoneal within 24 hours. It is particularly noteworthy that in one case (No. 22), inoculated intraperitoneally, the bacilli appeared in the blood within 10 minutes after inoculation. In view of these observations, there can be no doubt about the entrance of bacilli into circulating blood, in whatever way the inoculation occurs. The time at which the organism reaches the blood seems somewhat earlier than indicated by earlier workers. If a larger amount of blood had been cultured, the bacilli might have been found earlier.

APPEARANCE OF BACILLI IN URINE

To determine whether bacilli appear in the urine, 10 animals were used, which had been infected with plague bacilli. After death they were dissected with great care, and the bladder isolated from the surrounding tissues, washed with sterile salt solution and alcohol, punctured with a thin needle, and 0.2 c c. to 2 c c. of the urine withdrawn in 6 of the 10 guinea-pigs. Of every sample of urine, an equal quantity was poured into bile, citrated broth and agar medium and incubated.

As seen from table 2, 5 of 6 case gave positive results; in one sample contamination took place. The citrated broth gave the best result.

TABLE 2
BACILLI IN URINE

Number of Animal	Inoculation	Amount of Urine in C c.	Culture Mediums		
			Agar	Citrated Broth	Bile
9	Subcutaneous.....	1.5	+	+	0
10	Subcutaneous.....	2.0	0	+	+
16	Cutaneous.....	0.2	+	+	+
18	Cutaneous.....	0.5	0	+	+
19	Subcutaneous.....	2.0	..	Contaminated	
22	Intraperitoneal.....	0.5	+	+	0

APPEARANCE OF BACILLI IN BILE

Nine different samples of bile were obtained from animals used in the previous experiments. The gallbladder was ligated and cut off without injury to the liver and carefully washed many times in sterile salt solution. After three washings, no bacteria could be detected in the

solution used. By puncturing the bladders, from 0.1 to 1 c.c. of the bile was obtained and the three different culture mediums inoculated. In 6 cases, positive results were obtained.

TABLE 3
BACILLI IN BILE

Number of Animal	Inoculation	Amount of Bile in C c.	Culture Mediums		
			Agar	Citrated Broth	Bile
19	Subcutaneous.....	0.4	+	+	+
10	Subcutaneous.....	0.5	0	0	0
16	Cutaneous.....	0.1	+	+	0
17	Cutaneous.....	0.1	+	+	0
19	Subcutaneous.....	1.0	0	0	0
20	Subcutaneous.....	0.3	+	—	+
22	Intraperitoneal.....	0.3	0	0	0
23	Intraperitoneal.....	0.5	+	+	0
24	Intraperitoneal.....	0.3	0	0	+

SUMMARY

In whatever way *B. pestis* was inoculated, it entered the circulating blood in all of the animals.

Of 6 samples of urine 5 contained *B. pestis*.

On cultivation, bile, in 6 of 9 cases, gave growth of bacilli.

In view of these observations, plague apparently develops in much the manner that typhoid fever does. The invasion of bacilli into blood may occur earlier than generally recognized, and it may grow not only in tissues but also in blood itself. That bacilli pass out in the bile and urine seems quite probable.

PRECIPITIN REACTIONS OF SERUM PROTEINS

LUDVIG HEKTOEN AND WILLIAM H. WELKER

From the John McCormick Institute for Infectious Diseases and Laboratory of Physiological Chemistry, University of Illinois College of Medicine, Chicago

In this paper we report briefly the results of our work on the precipitin reactions of the euglobulin, pseudoglobulin and albumin of beef, dog, horse and human blood serum. These proteins were obtained in as pure form as possible by the method described.

Preparation of Proteins.—The blood serum is diluted with 2 volumes of distilled water, and a 33% saturated ammonium sulphate secured by adding a saturated solution drop by drop in a mixing machine. After standing 24 hours, the mixture is filtered. In the early stages of the work, an attempt was made to wash the precipitate with 33% ammonium sulphate, but this is not practical because of the tendency of the precipitate to go back into solution. The precipitate is drained as dry as possible, dissolved in 10% sodium chloride solution, the solution strained through cotton, and dialyzed in a collodion bag against distilled water under toluol. When the liquid in the bag no longer gives a test for the chloride ion, a relatively large amount of precipitate has come down, while the liquid contains a goodly quantity of water-soluble protein. The precipitate, suspended in a little of the solution, is centrifugated and washed in distilled water until the wash water no longer gives the biuret reaction. It is then redissolved in 10% sodium chloride solution and dialyzed again. When all of the chloride ion has been removed, the liquid again gives a positive biuret reaction. This process of purification of the precipitate obtained at 33% saturation is continued until on dialysis of its solution in a 10% sodium chloride solution to the complete removal of the chloride ion, the liquid, free from the precipitate, no longer gives a biuret reaction. The precipitate at this point is centrifugated and dried at 40 C. after being dissolved in the smallest possible amount of 10% solution of sodium chloride. If the purified protein is dried in the absence of electrolyte, it behaves like coagulated protein. This preparation is the euglobulin or true globulin.

The part that precipitates between 33 to 46% saturation of ammonium sulphate is the pseudoglobulin and is water soluble. This fraction is precipitated by raising the filtrate from the 33% saturation to 46% in the mixing machine. After standing 24 hours the solution is filtered, the precipitate drained as thoroughly as possible, and dissolved in distilled water. The solution is strained through cotton and dialyzed in a collodion bag against distilled water until free from the sulphate ion. Dialysis of solution of this first precipitate between 33 to 46% saturation gives large precipitates. At completion of the dialysis, the solution is filtered, saturated to 33% in a mixing machine and left standing for 24 hours. Usually, a small amount of precipitate occurs at this point. The solution is filtered, raised again to 46% and after 24 hours, filtered again, the precipitate redissolved and again dialyzed in a collodion bag against distilled water until free from the sulphate ion. In this second dialysis, a protein pre-

cipitate is always obtained, smaller in quantity than the first, but definite. This procedure is repeated until a solution of pseudoglobulin is obtained which gives no precipitate when dialyzed free from the sulphate ion against distilled water. The solution is evaporated to dryness at 40 C. under toluol.

The filtrate separated from the precipitate at 46% saturation is raised to 64% when a precipitate invariably occurs. This precipitate has not been studied. The filtrate of the 64% saturation is completely saturated with solid ammonium sulphate, and after standing over night, the precipitate is filtered off, dissolved in distilled water, dialyzed in a collodion bag against distilled water until free from the sulphate ion, and raised to 46% of ammonium sulphate in a mixing machine. Usually no precipitate occurs at this point on standing for 24 hours. The liquid is then raised to 64% in a mixing machine, when a large amount of precipitate forms. After standing for 24 hours, the mixture is filtered, the precipitate dissolved in distilled water, dialyzed in a collodion bag against distilled water until free from the sulphate ion, and dried at 40C. The filtrate of the 64% saturation is saturated with solid ammonium sulphate, and after standing over night filtered, dissolved in distilled water, dialyzed in a collodion bag against distilled water until free from the sulphate ion and dried at 40C. These two fractions are the albumins. The protein precipitating between 64 and 100% saturation with ammonium sulphate is the one used in our experiments.

TABLE 1
PRECIPITIN REACTIONS OF PROTEINS OF BEEF SERUM

Antigens	Serum of Rabbits Injected With			
	Euglobulin	Pseudoglobulin	Albumin	Serum
Euglobulin.....	32,000	200	0	2,000
Pseudoglobulin.....	10,000	100,000	0	10,000
Albumin.....	0	0	80,000	400
Serum.....	8,000	4,000	2,000	4,000

In tables 1-6, the figures are the highest antigen dilutions in contact with which the anti-serum formed a definite precipitate after one hour at room temperature.

In tables 1-3, lowest antigen dilutions with negative results, 1:100.

Preparation of Precipitin Serums.—Rabbits are immunized by intravenous injections. As a rule, 5 injections of increasing quantities are given 3-4 days apart. Of 1% solutions of pseudoglobulin and albumin in 0.85% salt solution, 1, 2, 3, 4, and 5 c.c. are injected in this way. Euglobulin in various strengths is dissolved in 10% salt solution, which is diluted to 1% just before injection, and injected in approximately the same quantities as pseudoglobulin and albumin. There were few failures in precipitin formation, and usually the rabbit serum was found rich in precipitin on the 4th day after the last injection. Occasionally 2 or 3 further injections of the antigen were given to increase the precipitin strength of the rabbit serum.

Tests.—Our tests have been made by the contact or layer method, and with progressive dilutions of the antigens, well beyond any possible inhibition zone in all cases, in order to determine the highest dilution of antigen giving precipitate after contact with the precipitin serum for one hour at room temperature. In the tables, the figures give the precipitin titers as determined in this way.

BEEF SERUM PROTEINS

In table 1 are given results of tests of beef serum and its proteins. One outstanding fact is that the serum albumin acted as a specific precipitinogen. There were no cross reactions between albumin and euglobulin or pseudoglobulin. The results in euglobulin and pseudoglobulin, on the other hand, indicate that these antigens were not absolutely free each from traces at least of the other. The pseudoglobulin precipitin serum, however, had only slight effect on euglobulin.

DOG SERUM PROTEINS

The results with dog serum proteins are illustrated in table 2, which shows that the albumin did not react with the precipitin serum for euglobulin or pseudoglobulin. The serum of rabbits injected with the albumin contained, however, some precipitins for euglobulin and pseudoglobulin, which may be explained on the score of the presence of

TABLE 2
PRECIPITIN REACTIONS OF PROTEINS OF DOG SERUM

Antigens	Serums of Rabbits Injected With				
	Euglobulin	Pseudo-globulin	Albumin	Serum	Laked Blood
Euglobulin.....	33,000	3,300	3,300	3,300	5,000
Pseudoglobulin.....	1,000	10,000	100	0	16,000
Albumin.....	0	0	10,000	0	1,600
Serum.....	1,000	1,000	1,000	8,000	1,600
Laked blood.....	20,000
Hemoglobin.....	40,000

minute traces of these proteins in the albumin fraction. Table 2 also indicates that the euglobulin contained some pseudoglobulin and that the pseudoglobulin contained some euglobulin. Finally, it may be pointed out that in the serum of a rabbit immunized with dog serum, precipitins for pseudoglobulin and albumin were not demonstrable, while in another rabbit immunized with laked dog blood, precipitins developed for euglobulin, pseudoglobulin, albumin, and hemoglobin.

HORSE SERUM PROTEINS

Table 3 illustrates results of tests of the proteins of horse serum. In this particular experiment, the 3 proteins conducted themselves as specific antigens in practically pure form, but in the case of other preparations such clean-cut separation was not obtained. In all instances of cross reactions, however, any particular precipitin could be removed

TABLE 3
PRECIPITIN REACTIONS OF PROTEINS OF HORSE SERUM

Antigens	Serums of Rabbits Injected With			
	Euglobulin	Pseudoglobulin	Albumin	Serum
Euglobulin.....	21,000	0	0	12,800
Pseudoglobulin.....	0	50,000	400	12,800
Albumin.....	0	0	25,000	12,800
Serum.....	2,000	2,000	1,600	800

readily from the antiserum by absorption with the proper antigen, and without materially reducing the strength of the antiserum in associated precipitins. An example of purification of precipitin serum by specific absorption is given in table 4.

TABLE 4
SPECIFIC ABSORPTION OF PRECIPITINS

Antigens	Serum of Rabbits Injected With						
	Euglobulin		Pseudoglobulin			Albumin	
	Original	After Mixing with Equal Quantity of Solution of Pseudo-globulin, and Removing by Centrifugation Precipitate that Formed on Standing	Original	After Mixing with Equal Quantity of Solution of Euglobulin, 1 : 3,000, and Removing by Centrifugation Precipitate that Formed on Standing	After Mixing with Equal Quantity of Solution of Albumin, 1 : 1,000, and Removing by Centrifugation Precipitate that Formed on Standing	Original	After Mixing with Equal Quantity of Pseudo-globulin, 1 : 1,000, and Removing by Centrifugation Precipitate that Formed on Standing
Euglobulin.....	33,000	24,000	3,300	0	0	0	0
Pseudoglobulin..	1,000	0	100,000	100,000	100,000	1,000	0
Albumin.....	0	0	100,000	100,000	0	1,000,000	400,000+

The lowest dilutions tested with negative results are 1 : 100 in case of pseudoglobulin and albumin, and 1 : 330 in case of euglobulin.

HUMAN SERUM PROTEINS

The proteins of human serum reacted (table 5) like the horse proteins, that is, in some of the experiments each protein appeared to be a specific antigen in pure or practically pure form, and in case of cross reactions there was no difficulty in separating the different precipitins by absorption according to the general scheme applied to precipitin serums for horse proteins (table 4).

IMMUNIZATION WITH SERUM OR WHOLE BLOOD

In view of the fact that euglobulin, pseudoglobulin, and albumin are distinct antigens, immunization with either whole blood or blood serum

should give rise to precipitins for each of these proteins. That this may happen is illustrated by tables 1, 2, 3, 5, and especially by table 6, which gives the results of tests with the serum of rabbits injected with human blood, with albuminous human urine, and with laked horse blood. Table 2 illustrates results with laked dog blood. Blood was laked by adding water, removing the corpuscular stroma by centrifugation, and then adding enough salt to give the normal salt strength. The details of the immunization are given in table 6, which also shows that in addition to precipitins for euglobulin, pseudoglobulin, and albumin, the serum of the rabbits, injected as described, with blood, contained also specific hemoglobin precipitins. It may be well to add that the hemo-

TABLE 5
PRECIPITIN REACTIONS OF PROTEINS OF HUMAN SERUM

Antigens	Serum of Rabbits Injected With			
	Euglobulin 987	Pseudoglobulin 1036	Albumin 1042	Laked Blood
Euglobulin.....	5,000	0	0	12,800
Pseudoglobulin.....	100	10,000	100	100,000
Albumin.....	0	0	100,000	200,000+
Serum.....	1,000	5,000	10,000	50,000

The lowest dilutions tested with negative results are 1:100 in case of pseudoglobulin and albumin, and 1:2,000 in case of euglobulin.

TABLE 6
EXAMPLES OF MULTIPLE PRECIPITIN FORMATION FOLLOWING INJECTIONS OF HUMAN BLOOD, ETC.

Antigens	Serum of Rabbit Injected Intra- venously with 1% Suspension of Citratd Blood, 5, 10, 15 c.c. One Day Apart, and Beginning on Sixth Day After Last Injection, with 5, 10, 15 c.c. of 5% Mixture of Laked Blood, also at Intervals of One Day	Serum of Rabbit Injected Intra- venously with 5% Solution of Laked Blood, 5, 10, 15 c.c. One Day Apart, Followed on the Sixth Day After the Last Injection by a Second Series of Such Injections	Serum of Rabbit Injected Intra- venously with Albuminous (Human) Urine, 4, 8, 12, 16 and 20 c.c. at 3-Day Intervals	Serum of Rabbit Injected Intra- venously with 3% Mixture of Laked Horse Blood, 5, 10, 15 c.c. One Day Apart, Followed on Sixth Day After Last Injection by a Second Series of Such Injections
	1	2	3	4
Laked blood.....	12,800	50,000	Not tested	16,000
Serum.....	6,400	50,000	12,800	1,000
Hemoglobin.....	8,000	25,000	0	8,000
Euglobulin.....	2,500	12,800	10,000	80,000+
Pseudoglobulin...	16,000	100,000	3,200+	32,000+
Albumin.....	8,000	200,000+	50,000	Not tested

Serum of rabbits 1, 2 and 3 tested with human antigens; serum of rabbit 4 tested with horse antigens.

globulin solutions used in these tests did not give any reactions with precipitin serum for euglobulin, pseudoglobulin or albumin.

SPECIES-SPECIFICNESS

As shown in table 7, the precipitin reactions of euglobulin, pseudoglobulin, and albumin of beef, dog, horse, and human blood serum obey the law of species-specificness so far as these 4 species are concerned. In no case did precipitin serum for the euglobulin, pseudoglobulin or albumin of any one of the 4 species react with any of these proteins from the other species in the dilutions tested. The lowest pseudoglobulin and albumin solutions tested were 1 to 100; the lowest euglobulin solutions tested were: beef, 1:200; dog and horse, 1:330; human, 1:2,000. Beef and human serums reacted to precipitin serum for horse euglobulin and pseudoglobulin, and beef serum to precipitin serum for human euglobulin, but only in low dilutions. No attempt to explain these reactions will be made at this time.

TABLE 7
SPECIES-SPECIFICNESS OF PRECIPITIN REACTIONS OF PROTEINS OF BEEF, DOG, HORSE
AND HUMAN SERUMS

Antigens	Serum of Rabbits Injected With											
	Beef			Dog			Horse			Human		
	Eug.	Pseu.	Alb.	Eug.	Pseu.	Alb.	Eug.	Pseu.	Alb.	Eug.	Pseu.	Alb.
Beef												
Euglobulin.....	+	±	0	0	0	0	0	0	0	0	0	0
Pseudoglobulin.....	+	+	0	0	0	0	0	0	0	0	0	0
Albumin.....	0	0	+	0	0	0	0	0	0	0	0	0
Serum.....	+	+	+	0	0	0	50	20	0	0	0	0
Dog												
Euglobulin.....	0	0	0	+	+	+	0	0	0	0	0	0
Pseudoglobulin.....	0	0	0	+	+	±	0	0	0	0	0	0
Albumin.....	0	0	0	0	0	+	0	0	0	0	0	0
Serum.....	0	0	0	+	+	+	0	0	0	0	0	0
Horse												
Euglobulin.....	0	0	0	0	0	0	+	0	0	0	0	0
Pseudoglobulin.....	0	0	0	0	0	0	0	+	±	0	0	0
Albumin.....	0	0	0	0	0	0	0	0	+	0	0	0
Serum.....	0	0	0	0	0	0	+	+	+	0	0	0
Human												
Euglobulin.....	0	0	0	0	0	0	0	0	0	+	0	0
Pseudoglobulin.....	0	0	0	0	0	0	0	0	0	±	+	±
Albumin.....	0	0	0	0	0	0	0	0	0	0	0	+
Serum.....	0	40	0	0	0	0	40	20	0	+	+	+

The figures mean positive reaction at that dilution of the antigen.
Eug. indicates euglobulin; Pseu., pseudoglobulin; Alb., albumin.

DISCUSSION

Our results indicate that the euglobulin, pseudoglobulin and albumin of blood serum are distinct and species-specific precipitinogens. In much

of the previous work ¹ on the precipitin reactions of these serum proteins, such definite evidence of precise, individual specifness in reaction was not obtained. Probably one important reason for this difference in results is differences in the purity of the preparations used in the experiments. At the same time, differences in the places of injection of the antigen as well as in the methods of testing may have been of influence also. In much of the earlier work now in mind, beef and horse proteins were studied. In 1909, Uhlenhuth ² wrote that up to that time definite differentiation of isolated proteins by specific precipitation had not been achieved, and that in his opinion the divergence in the reported results could be explained on the score of the use of impure preparations.

In the early work are many indications of specifness of the globulins, but consistent evidence of specifness of the albumin as well seems to have been obtained only by Leblanc ³ with beef proteins, by Taguchi ⁴ with human proteins, and by Oguchi ⁵ with horse proteins. Leblanc, whose work has been overlooked by many writers, found that the euglobulin, pseudoglobulin, and albumin of beef serum give distinct precipitin reactions and to differ also from beef hemoglobin. More recently, Taguchi obtained specific precipitins for human globulin and albumin, and these precipitins were species-specific except so far as globulin precipitins reacted also with monkey globulins. In this work Taguchi included beef, dog, cat, sparrow, guinea-pig, and goat proteins.

The demonstration by precipitin tests that serum proteins are distinct antigens is supported by the results that Dale and Hartley,⁶ Kato,⁷ and Doerr and Berger ⁸ obtained on studying the anaphylactic reactions of serum proteins, which they found followed the principles of individual and species-specificness, already shown by Wells and Osborne ⁹ to hold with certain vegetable proteins.

¹ Nolf: *Ann. de l'Inst. Pasteur*, 1900, 14, p. 297. Myers: *Centralbl. f. Bakteriol.*, I, 1900, 28, p. 237. Rostoski: *München. med. Wehnschr.*, 1902, 49, p. 740. Ascoli: *Ibid.*, p. 1409. Michaelis: *Deutsch. med. Wehnschr.*, 1902, 28, p. 733, 1904, 30, p. 1240. Oppenheimer's *Handbuch d. Biochemie*, 1910, 2, p. 552. Linossier and Lemoine: *Compt. rend. Soc. de biol.*, 1902, 54, pp. 85, 276, 320 and 369. Landsteiner u. Calvo: *Centralbl. f. Bakteriol.*, I, O., 1902, 31, p. 781. Umber: *Berl. klin. Wehnschr.*, 1902, 39, p. 657. Fuhrmann: *Beitr. z. chem. Phys. u. Path.*, 1903, 3, p. 417. Hunter: *Jour. Physiol.*, 1905, 32, p. 327. Ruppel: *Deutsch. med. Wehnschr.*, 1923, 49, p. 40. See also Uhlenhuth and Weidanz: Kraus and Levaditi, *Handbuch der Immunitätsf.*, 1909, 2, p. 721. Uhlenhuth u. Steffenhagen: *Kolle u. Wassermann's Handbuch*, 1913, 3, p. 257.

² Kraus-Levaditi: *Handbuch d. Immunitätsf.*, 1909, 2, p. 819.

³ La Cellule, 1901, 18, p. 335. Ide: *Fortschr. d. Med.*, 1901, 19, p. 234.

⁴ Kyoto Igaku-gasse, 1916, 13, p. 1.

⁵ *Jap. Med. World*, 1923, 3, p. 187.

⁶ *Biochem. Jour.*, 1916, 10, p. 408.

⁷ *Mitt. Med. Fakultät, Univ. Tokyo*, 1917, 18, p. 195.

⁸ *Ztschr. f. Hyg. u. Infektionskr.*, 1921, 93, p. 147; 1922, 96, p. 191 and 258.

⁹ *Jour. Infect. Dis.*, 1915, 17, p. 259.

Several observers have noted that serum albumin seemed less effective as antigen than the other serum proteins. Leblanc, however, found his anti-albumin (beef) serum more active with beef serum than the antisera for the other serum proteins. In our experiments the albumins apparently without any difficulty induced specific precipitins as readily as euglobulin and pseudoglobulin, but because we did not make repeated titrations of the serum of the immunized rabbits, we do not know whether the different precipitins describe similar curves as they pass into and out of the blood. Specific sensitization seems to develop later after injection of albumin than after injection of globulin (Dale and Hartley,⁶ Doerr and Berger)⁸ and it is suggested that in serum disease the different horse proteins may cause different rashes,¹⁰ sensitiveness to albumin being the last to develop. Oguchi⁵ found that on immunization with horse serum, globulin disappeared first from the blood of the rabbit and albumin last. He found evidence of at least 6 proteins in horse serum with distinct precipitinogenic properties.

Injection of rabbits with serum, blood or albuminous urine developed precipitins for the corresponding serum proteins as well as whole serum, and when hemoglobin was present in the antigen also for that hemoglobin. This result indicates that each protein in question retains its antigenic independence in the serum or blood, and that the fraction-specificness, as pointed out by Doerr and Berger⁸ in connection with their study of anaphylactic reactions, is not the result of artefact. As noted by Michaelis,¹ precipitins for all the proteins in a serum may not develop after injection of the serum. Undoubtedly rabbits vary greatly in their power to respond. As shown in table 2, dog serum in one case failed to call forth precipitins for dog pseudoglobulin and albumin, while in another experiment injections of laked blood called forth precipitins for the 3 proteins and hemoglobin also. Ascoli¹ obtained precipitins for beef euglobulin, pseudoglobulin and albumin by immunizing with beef serum, and the results of his absorption experiments pointed toward the presence of specific precipitins.

That there are many distinct antigens in blood and serum, and that consequently many distinct antibodies may arise from immunization with these complex antigens is recognized to bear directly on the question whether antigen and antibody can exist free side by side. It appears that in interpreting certain observations suggesting such coexistence, the rôle of multiple antigens and antibodies has not been taken into

¹⁰ Dale and Hartley (Reference 5). Coca: *Tice's Practice of Medicine*, 1920, 1, p. 168. Davidson: *Glasgow Med. Jour.*, 1919, 91, p. 321. 92, p. 20, 75, 129, 182. Hooker: *Jour. Immunol.*, 1923, 8, p. 469.

account sufficiently as a source of error, and Opie ¹¹ in his recent study on the relation of antigen to antibody in the blood, found no evidence of the free coexistence of a simple antigen (crystallized egg albumin) and its precipitin.

Because blood proteins react to specific precipitins, the precipitin test can be used to study the kinds and quantities of such proteins in the blood serum, arachnoid fluid, urine and other products. Doerr and Berger ¹² suggest that anaphylactic tests be made to determine the relative amounts of different proteins in the blood in various diseases; for practical purposes, the precipitin test seems easier of application. We find that the change in the protein content of horse plasma or serum after concentration of the antitoxic (diphtheria) globulin fraction can be determined by precipitin tests for horse euglobulin and albumin. The results of a few such tests are given in table 8. Hektoen and

TABLE 8
PRECIPITIN TESTS OF ANTIDIPHThERIA SERUM

Antidiphtheria Serum	Serum of Rabbit Injected with Horse		
	Euglobulin	Pseudoglobulin	Albumin
1. As separated.....	1,000	10,000	1,000
Concentrated.....	10,000	10,000,000	100
2. As separated.....	10,000	1,000,000	1,000,000
Concentrated.....	10,000	10,000,000	1,000
3. As separated.....	10,000	1,000,000	10,000
Concentrated.....	10,000	10,000,000	1,000
4. As separated.....	1,000	10,000	10,000
Concentrated.....	10,000	10,000,000	1,000

The figures indicate the approximate highest dilutions of antidiphtheria serum in contact with which the precipitin serums formed definite precipitates after one hour at room temperature.

Neymann ¹³ have shown that the precipitin test can be used to determine the amount of albumin or globulin in the arachnoid fluid. Precipitin tests of the urine in infections of children, especially diphtheria and scarlet fever, reveal that albumin may appear in the urine often without admixture with other proteins, and it may be of interest to study minutely the precipitin reactions of the urinary proteins in various conditions.

Wells ¹⁴ in particular has emphasized and illustrated the use of immunological methods in studying problems of protein chemistry. His thesis, abundantly supported by the facts at hand, is that immunologic

¹¹ Jour. Immunol., 1923, 8, p. 55.

¹² Ztschr. f. Hyg. u. Infektionskr., 1921, 93, p. 147.

¹³ Jour. Am. Med. Assn., 1920, 75, p. 1332; Jour. Nerv. and Ment. Dis., 1922, 56, p. 16.

¹⁴ Chemical Aspects of Immunity, in press.

specificity "depends on the chemical composition of the protein molecule which constitutes the antigen;" accordingly, the differences in the precipitin reactions of serum proteins depend on chemical differences, and it seems to us that the precipitin test may prove of increasing value as a means of assay and identification of individual proteins.

SUMMARY

Euglobulin, pseudoglobulin and albumin of beef, dog, horse and human serums are individually distinct, species-specific precipitinogens; in other words, each of these species has its own euglobulin, pseudoglobulin, and albumin, and within the species each of these proteins appears to be different from the other two.

Precipitin serums produced by injecting blood, serum, or albuminous urine, may contain specific precipitins for the corresponding serum proteins, and also for hemoglobin if it was present in the antigen. This result indicates that each protein in question exists as an independent, antigenic unit in the blood or serum, and that its antigenic individuality is not the artificial product of the process of separation.

ISOLATION OF *B. BOTULINUS*, TYPE B, FROM FECES BY USE OF BLOOD AGAR PLATES IN ANAEROBIC JAR

MARY W. WHEELER AND ELEANOR M. HUMPHREYS

*From the Division of Laboratories and Research, New York State Department of Health,
Albany*

Cases of botulism are reported in which bacteriologic evidence is lacking because specimens of the suspected food cannot be obtained. This report of the isolation of *B. botulinus* from the feces in a typical case of botulism is therefore of value in helping to establish the diagnostic significance of such examinations. It is also of interest in demonstrating the superiority of the use of blood-agar plates incubated in an anaerobic jar as a method for isolation and purification of anaerobes.

Meyer and Geiger¹ suggested the possibility of feces examination as an aid in the diagnosis of suspected cases of botulism. Other observers had previously demonstrated the presence of *B. botulinus* in the intestinal tract of human patients at necropsy, and in the excreta of animals suffering from botulism or after experimental feeding. Since constipation is an almost constant symptom of the disease, the remnants of the causative meal may be present in the intestines for some days. In 5 cases of botulism investigated by Meyer and Geiger, *B. botulinus* was isolated from the feces in 4 instances. In one outbreak in which none of the suspected food was available, *B. botulinus*, type A, was isolated from the feces of 2 of the patients on the 6th, 7th and 12th days of the disease. In the second outbreak, *B. botulinus*, type A, was isolated from the feces of one of the patients on the 6th day and also from the washings of the can containing the suspected food (spinach).

Graham and Barger² examined over 200 specimens of body excretions (feces and urines) from 80 cases of poliomyelitis and found *B. botulinus* in 1 specimen of feces and in 5 specimens of urines. Tanner and Dack³ report the isolation of *B. botulinus*, type B, from 2 of 10 specimens of feces from normal persons. The specimens were plated on dextrose-agar plates and incubated under anaerobic conditions. Geiger, Dickson and Meyer⁴ however, were unable to demonstrate the presence of this organism in 50 specimens of feces from normal persons who had ingested raw fruits and vegetables which had been shown to be contaminated with *B. botulinus* spores.

On June 7, 1923, this laboratory received a small amount of feces collected on a swab with a request for examination for *B. botulinus*.

Received for publication, May 9, 1924.

¹ U. S. Public Health Rept., 1921, 36, p. 1313.

² Bull. Univ. Ill., 1921, 7, p. 23.

³ Jour. Am. Med. Assn., 1922, 79, p. 132.

⁴ U. S. Public Health Bulletin, 1922, No. 127.

This specimen, taken on the 6th day after the first symptoms appeared, was submitted by Dr. Lang, Health Officer of the town of Cato, New York, at the request of Dr. C. R. Hervey of Oswego, New York, District State Health Officer, who was called as consultant and to whom we are indebted for the following report of the case.

On May 30, Mrs. W. was taken suddenly ill with explosive vomiting accompanied by bloating of the abdomen, but she apparently recovered quickly. Forty-eight hours later, however, she developed further symptoms typical of botulism—diplopia, ptosis of both upper lids, dilation of pupils, which were nonreactive to light, paralysis of the throat, difficulty in swallowing amounting almost to strangulation, obstinate constipation and difficult urination. There were no sweats nor chills, and no rise in temperature was observed until June 5, when it was 100.5 F. At this time, ability to swallow had returned to an appreciable degree, but the visual disturbances persisted. The mind remained clear throughout. There was no drowsiness, and general prostration was not marked. Recovery was slow, and a month later the patient was still under a physician's care.

The food responsible for this case was probably home-canned chicken, but none of it was available for examination. On May 29, the patient had opened a jar of chicken canned by herself, and had tasted the contents before cooking and serving it to the rest of the family. None of the family who ate the chicken only after it had been cooked became ill.

The specimen was emulsified in sterile salt solution. The amount of material submitted was so small that even with only 5 c c. of salt solution, the suspension obtained was very thin. Other workers had found that van Ermengen broth is unsuitable as a preliminary enrichment medium for the isolation of *B. botulinus*. It was decided, therefore, to use Hitchens' medium (0.2% dextrose infusion broth containing 0.1% agar), since this medium had been found favorable for the development of anaerobes. A 250 c c. bottle, containing 100 c c. of medium, was inoculated with 2 c c. of the suspension previously heated in a water bath for 1 hour at 60 C. The culture was incubated at 33-35 C. At the end of 24 hours, growth was heavy. In smears prepared from the culture, after 4 days' incubation, many gram-positive cocci, gram-negative bacilli and several types of gram-positive, spore-bearing bacilli, some with subterminal spores suggestive of *B. botulinus*, were seen. In order to separate the spore-bearing anaerobes from the cocci and gram-negative bacilli present, a portion of the culture was heated in a water bath for 1 hour at 70 C., and subcultures were made into Hitchens' medium and into chopped meat medium. After one week's incubation at 33-35 C., the supernatant broth from the culture in the chopped meat medium was

filtered and its toxicity tested. Two c.c. of this filtrate, inoculated subcutaneously, killed a 270 gm. guinea-pig in about 55 hours, with typical symptoms of botulism. A guinea-pig inoculated with the same dose of toxin and 300 units of type A botulinus antitoxic serum died in 50 hours, while the animal receiving 2 c.c. of toxin and 300 units of type B. botulinus antitoxic serum developed no symptoms.

The filtrate from a second subculture into meat medium, tested after 11 days' incubation, was still more toxic, 2 c.c. killing a guinea-pig in from 18 to 25 hours, while a subculture from the toxic meat culture grown for 18 days in van Ermengen broth contained no toxin.

A second specimen of 20 c.c. of intestinal washings taken on the 14th day of the disease was received on June 12. No toxin could be demonstrated in cultures inoculated from this specimen.

Repeated attempts, extending over a period of several months, were made to isolate a pure culture of *B. botulinus* from the toxic meat cultures. At least 18 series of dextrose infusion agar shake cultures were made, but from none of these was it possible to isolate *B. botulinus*. Only 3 series of these cultures contained colonies resembling those of *B. botulinus*. Fourteen transplants were made, but none of them proved to be *B. botulinus*. Later, attempts were made to isolate *B. botulinus* from these cultures by plate cultures grown in a hydrogen-anaerobic jar.

Hall,⁵ in addition to the usual deep agar dilution cultures, advises the use of blood-agar slant cultures grown under anaerobic conditions for isolation and purification of anaerobes, and describes the characteristic colonies of a large number of anaerobes on this medium. Zeissler⁶ found that the pathogenic, anaerobic, spore-bearing bacilli produced characteristic colonies on 2% dextrose infusion agar containing 15-20% blood. Richardson and Dozier,⁷ mention isolating delicate anaerobes by the use of sheep's blood-agar plates incubated in a hydrogen anaerobic jar, but give no details of the technic. Recently (since this work was begun) Wagner, Dozier and Meyer⁸ have described the characteristic colonies of *B. botulinus*, *B. sporogenes* and *B. tetani* on sheep's blood-agar plates incubated in a hydrogen anaerobic jar.

The toxic meat cultures were plated on dextrose infusion agar, chopped meat agar and on 2% extract agar plus 5% horse blood. Control cultures of *B. botulinus* were also made. The plates were incubated in a hydrogen-anaerobic jar at 33-35 C. for 48 to 72 hours. Growth was obtained on all the plates, but no marked variation in the type of the

⁵ Jour. Infect. Dis., 1922, 30, p. 445.

⁶ Ztschr. f. Hyg. u. Infektionskr., 1918, 86, p. 52. Kraus u. Uhlenhuth: Handb. d. mikrob. Technik, 1923 2, p. 961.

⁷ Jour. Infect. Dis., 1922, 31, p. 67.

⁸ Ibid., 1924, 34, p. 63.

colonies present was observed except on the blood-agar plates. Although these were heavily seeded, several different types of colonies were noted. The predominating type was a large, moist, raised, round or slightly irregular, opaque, white colony, usually slightly hemolytic. There were also a few well isolated, small, round or irregular, flat, transparent, grayish, hemolytic colonies resembling those on the botulinus control plate. Transplants from all types of colonies were made to dextrose semisolid agar. From one of the transplants made from a colony resembling *B. botulinus*, a pure culture of *B. botulinus*, type B, was obtained. Cultures of 4 other transplants from apparently similar colonies did not produce any toxin. Blood-agar plates, inoculated from 2 of these nontoxic cultures, contained no colonies resembling *B. botulinus*, while on the plates from the other 2 cultures, a few colonies resembling *B. botulinus* were found, but the predominating colony was a moist, raised, white colony resembling the predominating type of colony on the original plates.

A second series of blood-agar plates, inoculated from the toxic meat culture and incubated for 72 hours in the hydrogen-anaerobic jar, contained many well isolated colonies resembling those of *B. botulinus*. Four of these colonies were transplanted, and all produced potent toxins which killed guinea-pigs with typical symptoms of botulism. Blood-agar plates from these cultures contained only one type of colony.

The results of the use of extract-agar blood plates for separating *B. botulinus* from the contaminating organisms present in the original cultures were so successful that further experiments were made to determine whether different strains of botulinus produce a constant type of colony on this medium sufficiently characteristic to be readily distinguished from colonies of other anaerobes which might be associated with them. Since other workers have recommended infusion agar or a peptic-digest agar as the base for the blood-agar medium, a comparison was also made of the growth and colony formation of all organisms studied on (a) 5% horse-blood agar prepared with extract agar, (b) 5% horse-blood agar prepared with 0.5% dextrose extract agar, and (c) 5% blood agar prepared with 0.5% dextrose-infusion agar. The reaction of the agars used was P_H 7.4-7.6

Six toxic strains of *B. botulinus*, 3 type A strains—3 type B strains; 2 nontoxic strains of *B. botulinus* (received labeled *B. botulinus* but not found toxic); 2 strains of *B. sporogenes*; 2 strains of *B. histolyticus*; 1 strain of *B. putrificus*; 2 strains of *B. tetani*; and 1 strain of *B. welchii*

were studied. These organisms were plated repeatedly on the mediums, and the colony formation was studied after incubation of the cultures in a hydrogen-anaerobic jar at 33-35 C. for from 48 to 72 hours.

B. botulinus (*Toxic Strains*).—On medium A, 2 strains produced small, round or irregular, flat, moist transparent, hemolytic colonies which on moist agar became confluent. The other strains produced larger, round to slightly irregular, moist, raised, grayish white, semitransparent, hemolytic colonies. On mediums B and C, growth was usually more luxuriant, and all strains produced round or irregular, ameboid, raised, moist, grayish white, semitransparent colonies surrounded by a wide irregular zone of hemolysis. Older colonies tended to become spreading with flattened or depressed, transparent centers.

B. botulinus (*Nontoxic Strains*).—One of the nontoxic strains produced colonies similar to those of the toxic strains; the other strain, colonies similar to those of *B. sporogenes* as described below.

B. sporogenes.—On medium A, both strains produced large colonies having slightly raised, moist, grayish, semitransparent centers and wide, irregular, transparent, filamentous margins. The colonies were only slightly hemolytic. On mediums B and C, the colonies were similar but heavier, having large, moist, raised, opaque, white centers with grayish white, opaque, filamentous margins. Hemolysis was usually slight but more marked than on A.

B. histolyticus.—On medium A, one strain produced small, round or slightly irregular, raised, moist, grayish white, nonhemolytic colonies. The other strain produced colonies which were similar but usually very irregular or ameboid in shape. On mediums B and C, both strains produced colonies similar to those of the same strain on A, but growth was slightly heavier, and the colonies were usually larger and more opaque.

B. putrificus.—This strain did not grow on any of the mediums used until after the 4th or 5th day. On all mediums, the colonies were minute, round, raised, transparent nonhemolytic, later becoming slightly larger, dry and flattened with an irregular, mottled surface.

B. tetani.—Both strains studied grew poorly on all the mediums used. One strain produced only minute, raised, transparent, nonhemolytic colonies. The other strain produced colonies varying from minute colonies to larger colonies with fine, interwoven filaments extending from irregular, raised, transparent centers. The larger colonies were slightly hemolytic.

B. welchii.—On medium A, this strain produced large, round, slightly raised, moist, semitransparent, grayish, hemolytic colonies. The hemolytic zone was surrounded by a wide band of a deeper red than that of the uninoculated medium. On mediums B and C, the colonies were large, round, moist, raised, white and opaque. Hemolysis was much more marked than on medium A, and the zone of hemolysis was surrounded by a narrow band of intense red.

Blood-agar plates were also smeared with mixed cultures containing *B. botulinus*, *B. sporogenes*, and *B. histolyticus*. The three types of colonies characteristic for these species could be readily distinguished. The tendency for mixed colonies to form was noted, but there were many well isolated colonies which were apparently pure.

SUMMARY

The presence of botulinus toxin, type B, was demonstrated in cultures inoculated with a specimen of feces from a typical case of botulism taken on the 6th day of the disease.

Repeated attempts to isolate *B. botulinus* from the cultures in which toxin had been demonstrated by the use of deep agar-dilution cultures in dextrose-infusion agar were unsuccessful.

A pure culture of *B. botulinus*, type B, was readily isolated from the cultures in which toxin had been demonstrated, by the use of 5% horse-blood extract agar plates, incubated in a hydrogen-anaerobic jar.

The colony characteristics of a number of different anaerobes were studied on 5% horse blood agar, prepared with (a) extract agar, (b) 0.5% dextrose extract agar, and (c) 0.5% dextrose-infusion agar. On these mediums, different strains of the same species produced colonies with fairly constant characteristics which were typical of that particular species.

Most of the organisms studied grew readily on all mediums used, but growth was usually more luxuriant and the colony characteristics were more definite on mediums B and C.

While the addition of 0.5% dextrose to the extract agar used for the blood agar stimulated growth in some instances, the use of dextrose-infusion agar had no distinct advantage over the dextrose-extract agar.

FOOD ACCESSORY FACTORS (VITAMINES) IN BACTERIAL GROWTH

IX. GROWTH OF SEVERAL COMMON BACTERIA IN A SYNTHETIC MEDIUM AND RELATION OF SUBSTANCES FORMED BY THEM TO GROWTH OF YEAST

R. C. ROBERTSON

*From the Department of Pathology and Bacteriology, University of Illinois College of
Medicine, Chicago, Ill.*

In a previous paper,¹ it was pointed out that washed yeast cells were incapable of continued growth on a synthetic medium when transplants were made every 48 hours. More recently, it was shown² that *B. coli* not only was capable of continued growth on this medium, but also apparently generated some substance or substances during growth which permitted the continued growth of washed yeast cells when planted in the filtrate. We have made no endeavor as yet to identify these substances by animal feeding or other experiments, but they seem to have a growth stimulating effect on yeast cells and are therefore apparently of a vitamin-like nature.

As mentioned,² a review of the literature indicates that some growth accessory substances apparently are formed by bacteria in the soil, from which they are taken over by the plants and so eventually by the animal kingdom. Our previous work tends to confirm the findings of other investigators along these lines, and it was with the hope of further substantiating and adding to these results that the following experiments were made.

A medium was prepared from chemically pure substances as follows: asparagin (Merck) 3.4 gm., calcium chloride 0.1 gm., dextrose 20.0 gm., magnesium sulphate 0.2 gm., potassium phosphate (K_2HPO_4) 1.0 gm., sodium chloride 5.0 gm., sterile distilled water to 1 liter. These substances were dissolved by heating the mixture to 100 C., the reaction adjusted to P_H 7.4, autoclaved at 20 pounds steam pressure for 30 minutes, tubed in 5 c.c. lots and again autoclaved at the same temperature for an equal length of time. All glassware employed had previously been thoroughly cleansed, washed with N/1 NaOH, rinsed in sterile distilled water, and autoclaved at 20 pounds steam pressure for 30 minutes.

Received for publication, May 12, 1924.

¹ Jour. Infect. Dis., 1923, 32, p. 152.

² Ibid., 1924, 34, p. 395.

The following organisms were employed because of their frequent occurrence in nature, and also to observe possible differences existing between pathogenic and nonpathogenic bacteria when grown in this medium:

B. coli communis, *B. diphtheriae*, *B. dysenteriae*, *B. proteus*, *B. pyocyaneus*, *B. subtilis*, *B. typhosus*, *B. paratyphosus* A and B, *Sarcina lutea*, *Staph. albus* and *aureus*, *Spirillum cholerae*, *Sp. metchnikovi*, *Saccharomyces cerevisiae* (baker's yeast).

Two strains of each organism were obtained, and each strain was tested separately. No noteworthy variation in results was observed. All were grown for 3 generations on nutrient broth, with the exception of yeast, which was grown on dextrose broth, with transplants every 24 hours. At the end of this time, tubes of the synthetic medium were inoculated with one loop of the broth culture (about 1/30 c.c.) of the organism in question. Transplants were made every 48 hours, the cultures being incubated at 37 C. in the interim. Controls were made in nutrient broth, except yeast, which was grown in dextrose broth.

The results shown in table 1.

TABLE 1
GROWTH ON SYNTHETIC MEDIUM

Organism	Continued Growth
<i>B. coli communis</i>	+
<i>B. diphtheriae</i>	0
<i>B. dysenteriae</i>	0
<i>B. prodigiosus</i>	+
<i>B. proteus</i>	+
<i>B. pyocyaneus</i>	+
<i>B. subtilis</i>	+
<i>B. typhosus</i>	0
<i>B. paratyphosus</i> A and B.....	0
<i>Sarcina lutea</i>	+
<i>Staph. albus</i> and <i>aureus</i>	0
<i>Sp. cholerae</i>	0
<i>Sp. metchnikovi</i>	0
<i>Saccharomyces cerevisiae</i> (baker's yeast).....	0
Controls (nutrient broth).....	+

Where continued growth is recorded, transplants had been carried through the 50th generation in apparently undiminished luxuriance. Where continued growth is negative, the duration of growth at no time exceeded 8 generations.

Continued growth was judged both by the appearance of the culture and the result of test inoculations on dextrose and nutrient agar, using 1 loop of the inoculated medium in all cases.

Control against contaminations was frequently made by use of nutrient agar plates, endoplates, and stained smears.

The 51st generation of all the organisms for which continued growth is recorded was transplanted to a flask containing 1 liter of the synthetic medium, and placed in the incubator at 37 C. for 10 days. At the end of this time, the inoculated mediums were separately passed through Berkefeld filters, which had previously been prepared by passing through it 250 c.c. of N/1 NaOH followed by 750 c.c. of sterile dis-

tiled water, and autoclaved at 20 lbs. steam pressure for 30 minutes. These mediums were then titrated to P_H 7.4, tubed in 5 c.c. lots and autoclaved at 20 lbs. steam pressure for 30 minutes.

Washed yeast cells were then prepared by transplanting every 24 hours for 3 days in dextrose broth, then throwing down in a centrifuge 3 times with sterile distilled water.

Tubes of the filtrate mediums as prepared, together with dextrose broth as controls, were now inoculated with 1 loop of the washed yeast suspension. Transplants were made every 48 hours through 50 generations. Growth was tested by frequent inoculation of dextrose agar slants. No contaminations occurred. The results are shown in table 2.

TABLE 2

RESULTS OF INOCULATION OF SYNTHETIC MEDIUM FILTRATE WITH WASHED YEAST CELLS
THROUGH 50 TRANSPLANTS

Synthetic medium plus <i>B. coli</i> (filtered).....	Continued growth
Synthetic medium plus <i>B. prodigiosus</i> (filtered).....	Continued growth
Synthetic medium plus <i>B. proteus</i> (filtered).....	Continued growth
Synthetic medium plus <i>B. pyocyaneus</i> (filtered).....	Continued growth
Synthetic medium plus <i>B. subtilis</i> (filtered).....	Continued growth
Synthetic medium plus <i>Sarcina lutea</i> (filtered).....	Continued growth
Controls (dextrose broth).....	Continued growth

DISCUSSION

Analysis of the technic indicates that any growth stimulating substance carried over by the first inoculation would before the end of the experiments be diluted to such an extent as to be practically negligible. Therefore, it appears that the growth of any organism on the original synthetic medium would indicate either that growth stimulating substances differing from those required by yeast were present and fulfilled the demands of the organism in question, or else that organisms so grown were either capable of continued growth without the aid of accessory growth substances or formed these substances during their growth on the medium in question.

We believe that the latter point of view appears most reasonable and that the organisms whose growth was continuous on this medium formed during their growth thereon substances which are of the nature of growth stimulating substances. Whether these substances are products common to all the organisms employed in these experiments or are identical, we are unprepared to say. All do at least contain the substance or substances apparently essential to the growth of yeast on the medium employed. Further work in animal feeding is required to

establish the relationship of these substances to any of the recognized vitamins.

It is to be noted that all the organisms producing these substances belong to the nonpathogenic or the slightly pathogenic organisms continuous on this medium. But we cannot because of this fact conclude that the latter are incapable of continuous growth on, or of producing growth stimulating substances in, all vitamin-free medium. It is true that as a rule the more virulent bacteria are more sensitive and are also more fastidious as to their nutritive requirements.

SUMMARY

The synthetic medium employed in the experiments reported permits the continued growth of several common varieties of bacteria, but other varieties chiefly pathogenic and also *Saccharomyces cerevisiae* soon died after a few transplants in this synthetic medium.

After growing the several varieties through 50 generations in the synthetic medium, they were grown in a liter of this medium for 10 days. The medium was then filtered, titrated, tubed, and autoclaved. On this medium yeast now continued to grow through 50 generations.

IS ABILITY TO UTILIZE CITRATE READILY ACQUIRED OR LOST BY THE COLON- AEROGENES GROUP?

STEWART A. KOSER

From the Department of Bacteriology, University of Illinois, Urbana

In a previous study of the differential tests employed to separate the several sections within the colon-aerogenes group, it was found¹ that the test of citrate utilization correlated more closely with the source of the cultures than did the usual methyl red and Voges-Proskauer tests. Also, when applied to cultures obtained from water supplies of different sanitary quality, the citrate test gave results correlating more closely with the sanitary survey than did the other differential tests.² Since there is some evidence, therefore, that the citrate test may be of practical value in the examination of water supplies, it would seem advisable to secure more data concerning what may be termed the stability of the citrate utilizing powers of the various colon group organisms.

Ordinarily, typical *Bact. coli* when isolated from the feces of man or lower warm-blooded animals refuses to utilize citrate and in a synthetic citrate medium fails to develop. On the other hand, the representatives of the colon-aerogenes group obtained from soil almost invariably developed in the citrate medium. Since the literature on the biochemical activities of bacteria contains many references to variability, the question may be raised whether typical fecal *Bact. coli* ever acquires the power to utilize citrate outside of the body, or, conversely, whether the soil types including *Bact. aerogenes* ever lose their citrate-utilizing ability. In the first paper,³ of the present series it was reported that colon group cultures kept on plain agar in the laboratory stock collection did not vary in their deportment in the citrate medium. Also, several aerogenes strains were transferred through numerous successive cultures in the citrate medium without any apparent diminution in the rapidity or luxuriance of growth. Several additional lines of work seem desirable, however, before a definite conclusion may be drawn

Received for publication, May 21, 1924.

¹ Koser, S. A.: *J. Bacteriol.*, 1924, 9, p. 59.

² Koser, S. A.: *J. Infect. Dis.*, 1924, 35, p. 14.

³ Koser, S. A.: *J. Bacteriol.*, 1923, 8, p. 493.

regarding the stability of the citrate-utilizing power. In the first place, do the intestinal *Bact. coli* cultures ever acquire the ability to attack citrate after a prolonged sojourn in water or in soil? Also, may *Bact. aerogenes* cultures ever lose their ability to utilize citrate when grown in an environment entirely different from that in which they were originally found and which simulates as nearly as possible the habitat of the intestinal *Bact. coli*?

The experiments reported in the present paper were designed to throw some light on the foregoing questions, and also to record further the effect of laboratory cultivation on the citrate-utilizing ability of a rather large collection of colon-aerogenes cultures.

BEHAVIOR TOWARD THE CITRATE TEST AFTER A SOJOURN
IN WATER

To determine whether the ability to utilize citrate may be altered after a period of survival in water, samples of various waters were sterilized in the autoclave and experimentally inoculated with a number

TABLE 1
SUMMARY OF COLON-AEROGENES CULTURES USED TO INOCULATE WATER SAMPLES

Number of Cultures	Source	Differential Tests		
		Methyl Red	Voges- Proskauer	Citrate
10	Feces, human and animal.....	+	0	0
11	Soil.....	+	0	+
8	Soil.....	Var.*	Var.*	+
10	Soil.....	0	+	+
Total 39				

* Variable, changed from methyl red positive and Voges-Proskauer negative to the reverse of this on laboratory cultivation shortly after isolation. A description of these organisms has been given elsewhere (Koser: *J. Bacteriol.*, 1924, 9, p. 59).

of representative cultures. In all, a total of 39 colon-aerogenes group cultures was employed. The source of the cultures and the type, as shown by the differential tests, are given in table 1. The intestinal *Bact. coli* was represented by 10 strains of human or animal origin. The cultures from soil fell into several subgroups; in addition to the aerogenes type, there were 11 methyl red positive, citrate positive cultures and 8 cultures which varied in their deportment toward the methyl red and Voges-Proskauer tests. The deportment of the methyl red positive, citrate positive type was deemed especially interesting, since these organisms may be considered as intermediate between the intestinal *Bact. coli* and the soil aerogenes section.

Two different samples of natural waters were used in the course of these experiments. Both of them had a high mineral content and alkaline reaction, one being about P_H 7.4 to 7.6, while the hydrogen-ion concentration of the other was P_H 7.8 to 8.0. In addition to these, a solution of balanced M/500 phosphates in which the H-ion concentration was adjusted to P_H 7 was also used with several of the cultures. In each case, the water was filled into large test tubes, about 25 c.c. per tube and sterilized in the autoclave. The sterilized water samples were then inoculated from 24-hour agar slant cultures by removing a small amount of the growth, care being taken not to transfer any of the medium. Plate counts made immediately after inoculation showed the suspensions to contain usually from one to two million cells per c.c. All suspensions were then held in the dark at a temperature of from 18 to 22 C. for periods of from 3 to 4½ months.

At the end of this period, the surviving organisms were tested in the citrate medium, and their behavior at this time was compared with their previous deportment toward citrate. The tests of citrate utilization were made in several ways. The first method did not resort to intermediate cultivation between the water suspension and the differential tests. A loopful from each of the suspensions was transferred to a tube of citrate medium, and at the same time another loopful was also transferred to lactose broth to confirm the presence of viable organisms. In one instance, all of the organisms had apparently died; in all of the remaining suspensions, however, viable organisms were present as shown by the gas production in lactose broth. In every case, the tubes of citrate medium inoculated from the water suspensions of the soil cultures showed a moderately heavy growth within 3 days at 30 C., while those inoculated from the suspensions of fecal *Bact. coli* cultures all remained clear at this time. These tests were held for 4 weeks at 30 C. before discarding, in order to detect any delayed citrate utilization by *Bact. coli* from the water suspensions. In two tubes, a light turbidity was apparent after 4 weeks; all others remained negative. When subcultures were made from these two tubes into fresh citrate medium, no growth was apparent even after 4 weeks. Evidently, then, these two exceptions did not represent a real ability to develop continuously in the medium. Apparently, the citrate-utilizing ability of the various cultures was little changed, if at all, by the sojourn in water.

In addition to the direct inoculation, as just outlined, the suspensions were also plated on plain agar, and from the resultant colonies inocula-

tions were made into the citrate medium. Usually from 10 to 20 colonies of each culture were transferred, and altogether 549 such inoculations were made from individual colonies. In each case, the deportment in the citrate medium was similar to that shown before the period in water, and no change was ever observed.

INTESTINAL BACT. COLI IN SOILS

Twenty cultures of Bact. coli of intestinal origin, both human and animal, were used to inoculate two series of soil samples. One was

TABLE 2
EFFECT OF A 4 MONTHS' PERIOD IN STERILIZED SOIL ON CITRATE UTILIZING ABILITY OF
FECAL BACT. COLI; ALL STRAINS CITRATE NEGATIVE AT BEGINNING
OF EXPERIMENT

Culture Designation	After 4 Months in Soil		
	Lactose Fermentation	Growth in Citrate Medium	Number of Cells Inoculated (1 Loopful) as Detected by Plate Count
Human 1.....	+	0*	17,000
Human 2.....	+	0	3,200
Human 10.....	+	0	400
Human 12.....	+	0	170
Human 14.....	+	0	600
Human 16.....	+	0	950
Human 18.....	+	0	950
Human 20.....	+	0	570
Human 24.....	+	0	1,400
Human 25.....	+	0	100
Human 30.....	+	0	250
Rabbit 1.....	+	0	360
Rabbit 4.....	+	0	960
Rabbit 14B.....	+	0	5,000
Cat 1.....	+	0	160
Cat 3C.....	+	0	1,300
Sheep 4B.....	+	0	12,000
Guinea-pig 12.....	+	0	3,000
Rat 10.....	+	0	15,000
Dog 4A.....	+	0	1,500

* Held at 30 C. Results in citrate medium recorded at 3 days and also after 21 days.

a cultivated garden soil, P_H 6.6 to 6.8; the other was a forest soil having a P_H of 7.5 to 7.8. The soil samples were put into medium sized test tubes, filling the tubes about half full, and then sterilized, either in the autoclave or in the hot air oven. Cultures of the organisms for inoculation were prepared by growing for 24 hours on agar slants, and then suspending small amounts of this growth in sterile distilled water. The suspensions were adjusted to a slight turbidity which on plating gave an average of 15 to 25 million cells per c c. One c c. of each suspension was added to a tube of sterilized soil, and the samples were then stored in the dark at a temperature of from 18 to

22 C. for a period of about 4 months. Small amounts of sterile distilled water were added several times to make up the loss due to evaporation and to keep the soil moist.

At the end of the 4 months' period, small amounts of soil were added to tubes containing from 3 to 4 c.c. of sterile water, thoroughly shaken and after a brief period to allow the larger particles to settle to the bottom, one loopful of the resulting suspension was transferred to each of the following: (1) citrate medium, (2) lactose broth, and (3) a tube of melted agar, which was immediately plated. Thus it was determined whether the lactose fermenters had survived the period in the soil, and, if so, the approximate number of cells transferred in one loopful of the suspension, and whether growth occurred in the citrate medium following such inoculation. By this procedure, there was no intermediate plating on plain agar, and the same cells which had been in the soil were inoculated into the citrate medium and lactose broth. Since the two series of soil samples gave similar results, an outline of only one experiment is given in table 2.

Here it is seen that intestinal *Bact. coli* survived the 4 months' period in soil; all cultures fermented lactose promptly, and all refused to grow in the citrate medium. The inoculum which the various citrate tubes received varied from 100 to 17,000 cells. In another experiment, this ranged from 3,000 to 40,000 cells. In every instance, the organisms refused to develop, and it seems quite evident that the fecal *Bact. coli* did not acquire the ability to attack citrate, and that its nutritive requirements in this respect were unchanged.

SOIL CULTURES IN STOOL SUSPENSIONS

This experiment was designed to determine whether *Bact. aerogenes* and other soil types of the colon group might lose their ability to attack citrate after multiplication in an artificial environment somewhat similar to the habitat of *Bact. coli*. A dilute suspension of feces was made in water, tubed in test tubes in 10 c.c. amounts and sterilized in the autoclave. The sterilized suspensions were then inoculated with various cultures originally isolated from soil samples. Fourteen strains of *Bact. aerogenes* and 6 of the methyl red positive, citrate positive type were used. At 37 C., these 20 cultures were all capable of growing, at least to a certain extent, in this environment, and could be carried through successive transfers. They were transferred at weekly intervals for 4 months, then streaked on plain agar plates, and the resultant colonies

used to inoculate tubes of citrate medium. From 10 to 15 colonies of each of the 20 cultures were used, so that altogether 279 tubes of citrate medium were inoculated.

Cultivation in the feces suspensions seemed to have little if any effect on the citrate utilizing powers of these organisms, with perhaps one exception. In this case, cultures in the citrate medium exhibited a slower development than that shown by the original culture. The original culture utilized citrate readily, and produced a moderately heavy growth within 3 days at 30 C. The subcultures of this one strain when recovered from the feces suspension usually produced only a slight turbidity after 3 days, and in two instances showed no visible growth at this time. In these two cases, however, growth was apparent after 4 or 5 days, while by the 6th day all cultures exhibited a moderately heavy turbidity. All of the subcultures of the other 19 strains developed readily in the citrate medium.

TABLE 3
SUMMARY OF TYPES OF COLON GROUP ORGANISMS CULTIVATED THROUGH SUCCESSIVE
TRANSPLANTS IN CITRATE MEDIUM

Number of Cultures	Type	Source	Differential Tests		
			Methyl Red	Voges- Proskauer	Citrate
6	Bact. aerogenes.....	Soil	0	+	+
1	Bact. cloacae.....	Soil	0	+	+
19	(1).....	Soil	+	0	+
8	(2).....	Soil	Variable	Variable	+
Total 34					

(1) Differed from the intestinal Bact. coli in the ability to utilize citrate.

(2) Resembled aerogenes section in various culture and fermentation characteristics.

SUCCESSIVE TRANSPLANTS IN CITRATE MEDIUM

To test the ability of Bact. aerogenes and other citrate utilizers to sustain continued multiplication in the citrate medium, representative cultures were selected and run through 50 successive transplants in the usual medium of inorganic salts and 0.2 per cent. sodium citrate. Transfers were made with a small loop and at intervals of either 24 or 48 hours, depending on the rapidity of growth of the different strains. In all, 34 cultures were tested in this way, and all were found to be capable of continued multiplication through repeated transfers. At the completion of the 50th transfer, no decrease in either the rapidity or luxuriance of growth had been apparent at any time. A summary of the organisms used in these experiments is given in table 3.

RESULTS OF CITRATE TEST AT DIFFERENT INTERVALS DURING
LABORATORY CULTIVATION

As an additional check on the reliability of the citrate test when applied at different intervals, the entire collection of colon-aerogenes cultures was tested several times in the usual-citrate medium. These cultures were originally obtained from fecal specimens, from soil and from various water samples. After isolation, they were kept in the icebox on plain nutrient agar slants, and were transferred at intervals of about a month or 6 weeks. These cultures were kept in stock for periods varying from 6 months to 2 years, and during this period they were run through each of the differential tests at least twice, and in most cases 4 or 5 times, or occasionally more. A summary of the various cultures used, their source, and their behavior toward the differential tests, is given in table 4.

TABLE 4
STOCK COLON-AEROGENES CULTURES TESTED AT DIFFERENT INTERVALS IN CITRATE MEDIUM

Number of Cultures	Source	Differential Tests		
		Methyl Red	Voges- Proskauer	Citrate
81	Feces, water, soil (2 cultures).....	+	0	0
55	Water, soil, feces (2 cultures).....	+	0	+
1	Water.....	+ Var.	0	+
1	Feces (dog).....	+	0	Slow +
8	Soil.....	Var.	Var.	+
66	Soil, water, feces (few).....	0	+	+
1	Bottled mineral water.....	0	+	0*
Total 213				

Var. indicates variable results given by these tests at different times.

* This culture, on subsequent tests, gave a + result in citrate medium. This was the only instance in which any variability in the citrate test was noted.

The results of this work may be summarized briefly by stating that all cultures, with one exception, showed remarkably constant results in the citrate medium, and, as far as could be discerned, there was no tendency either to acquire or to lose the ability to attack citrate. The one exception occurred in the case of a culture originally isolated from bottled mineral water. This culture apparently belonged to the aerogenes section, for it was consistently methyl red negative and Voges-Proskauer positive, although when tested in the citrate medium it refused to develop. Thus far, this has been the only instance in which a member of the aerogenes section has failed to develop in the citrate medium. When tested again in the citrate medium, about a year after isolation,

a scanty slow development occurred, and similar results were given at different times thereafter. It should be noted that this is a change from the abnormal to the normal behavior for this type.

SUMMARY

The citrate differentiation, to separate the intestinal *Bact. coli* from other members of the colon group, appears to be quite constant and reliable. The ability to utilize citrate is apparently a fairly stable character, and evidently is not readily acquired or lost.

STUDIES ON RESPIRATORY DISEASES

XVIII. THE RELATIVE RELIABILITY OF THROAT SWABS FOR ISOLATING THE CAUSATIVE PNEUMOCOCCUS TYPE *

FRANK B. KELLY AND HARRY GUSSIN

From the Department of Hygiene and Bacteriology, University of Chicago

The introduction of antipneumococcus serum for lobar pneumonia therapy has made the immediate determination of the type of the causative pneumococcus organism of importance.

A number of methods have been introduced for the isolation of the pneumococcus, the most commonly used being mouse inoculation, Avery's blood broth and Oliver's precipitin test. Most workers have agreed that mouse inoculation is the most reliable.

In the mouse method, as well as in the others, the preliminary part of the process consists in obtaining sputum raised from the chest. Many observers definitely stress the importance of obtaining noncontaminated sputum.

Avery¹ states that care is to be taken in collecting sputum from the deep air passages to avoid saliva contamination. Vaughan² lays down the following regulation for the typing of pneumococci: "The laboratory worker should not accept a sputum specimen that is contaminated with saliva. The sputum specimen must be raised from the chest and not contaminated by saliva. And if the patient finds it impossible to raise sputum while lying on his back, he can often be made to cough while lying on his unaffected side." Clough³ considered that without other evidence type 3 and type 4 fished from sputum cultures that were not pure had no necessary etiologic relationship to the pneumonia. Nichols⁴ and McClelland⁵ also emphasized the desirability of getting a satisfactory specimen of sputum from the deep air passages.

Received for publication, May 28, 1924.

* This is one of a series of studies carried out in connection with the Influenza Commission established and financially aided by the Metropolitan Life Insurance Company of New York. Part of the expense of these studies has been met by a grant from the University of Chicago.

¹ Jour. Am. Med. Assn., 1918, 70, p. 17.

² Ibid., p. 431.

³ Bull. Johns Hopkins Hosp., 1917, 28, p. 306.

⁴ Military Surgeon, 1917, 31, p. 149.

⁵ Cleveland Med. Jour., 1918, 17, p. 226.

Contamination from the mouth is, however, not universally feared. Vaughan and Schnabel⁶ state that "It is improbable that a type 1, 2 or 3 organism would be concealed by a type 4 mouth organism on three successive cultivations. This is borne out by the fact that the organism found in pure culture in each pleural exudate has always been of the same type as that found in the sputum." Avery¹ considers that the disease-producing type of pneumococcus rapidly outgrows the less pathogenic variety, and is therefore rapidly demonstrable in the peritoneal exudate and heart blood of the inoculated mouse.

It is well known that coughing is an aggravating and distressing symptom of the disease. It is usually accompanied by a pain in the side, and at first is commonly hard, dry, and without any expectoration. Sometimes it is only after the crisis that the expectoration is more easily expelled; the sputum is often tenacious and has to be wiped from the lips of the patient. In children, it is almost impossible to obtain sputum.

We have made observations in 136 cases of lobar pneumonia to determine the reliability of throat swabs as compared with raised sputum and blood culture from the same patient. The throat smears were made from the back of the pharynx and around the tonsillar region. The swab was immediately placed in a tube of blood broth containing about 10 c.c. of plain broth, P_H 7.8, to which about 0.5 c.c. of sterile sheep blood had been added; the tube was incubated for from 4 to 6 hours, and about 1 c.c. of the supernatant fluid injected into a white mouse. At the same time, sputum obtained from the same patient was washed and injected directly into a mouse. Necropsy examination was performed on the mice, and the agglutination-precipitation test was performed similarly in both cases. Plates were usually streaked from the peritoneal exudate and heart blood. Confirmatory tests were made in doubtful cases.

For blood examination, from 1 to 2 c.c. of the patient's blood was drawn into a tube of dextrose broth and incubated for from 6 to 8 hours. When growth was present, 1 c.c. of supernatant fluid was transferred to a fresh tube of dextrose broth, which was incubated, and an agglutination test made on the new growth. This was done in order to prevent the interference of the serum in the bile test. The New York State Board of Health serum was used for typing (tables 1 and 2).

⁶ Arch. Int. Med., 1918, 22, p. 440.

It is evident from the data presented in the tables that throat swab cultures give practically the same results as sputum examinations. The method is of value in cases in which sputum cannot be raised or blood be drawn.

A throat swab represents the easiest way of obtaining a culture from a pneumonia patient. From the onset of the disease to the crisis, the patient may be too sick to cough; it is often too distressing and too painful to the patient to have him turn on his side to raise the sputum;

TABLE 1
RESULT OF AGGLUTINATION TESTS

Organisms	Throat Swabs, Number of Positives	Sputums, Number of Positives	Ratio of Positive Throat Swabs to Positive Sputums
Type 1 pneumococcus.....	30	33	0.91
Type 2 pneumococcus.....	24	25	0.96
Type 3 pneumococcus.....	5	6	#
Type 4 pneumococcus.....	53	51	1.04
Staphylococcus.....	4	2	#
B. Friedländer.....	6	5	#

Number of isolations too few to be of significance.

TABLE 2
CASES IN WHICH A POSITIVE BLOOD CULTURE WAS OBTAINED

Organisms	Throat Swabs, Number of Positives	Blood Cultures, Number of Positives	Ratio of Positive Throat Swabs to Positive Blood Cultures
Type 1.....	6	6	1.00
Type 2.....	9	9	1.00
Type 3.....	0	0	—
Type 4.....	14	15	0.93
Strep. hem.	1	1	#

and the cough is unproductive in some cases. It is also annoying to the attendants to handle the sputum and to wipe the patient's mouths. Sputums may be mixed in handling a number of cups. The blood culture is negative in some cases. A throat swab will give fairly reliable results.

The effect of the presence of mouth organisms on the causative pneumococcus organisms was found to be of slight importance, as shown by a comparative study of 45 samples of sputum from cases of lobar pneumonia. The sputums were injected into mice in washed and unwashed condition. The washing was done in the usual way; 3 rinsings in normal salt solutions followed by emulsification. An

agglutination and a precipitin test were made on every peritoneal exudate and followed by a confirmatory test. It was found that the washing of the sputum did not give any higher proportion of positive results or show any differences in the types found. By each method there were found six strains of type 1, nine of type 2, two of type 3, twenty-two of type 4 and six bile-insoluble organisms.

It was further found that when 1 c.c. of sputum containing a fixed type of pneumococcus is diluted as high as 1:10,000 (and in some cases as high as 1:100,000), and 1 c.c. of the latter dilution injected into a mouse, the type organism can still be recovered from the peritoneal exudate and heart blood. When normal sputums or known relatively avirulent type 4 cultures are mixed with the above mentioned dilutions and injected into mice, the fixed type of organism outgrows the other bacteria and can be easily recovered from the peritoneal exudate.

SUMMARY

Throat swabs were found to be reliable for the determination of the type of the causative pneumococcus organism.

A throat swab is relatively easy to make and prevents discomfort to the patient.

A throat swab is of special value in obtaining pneumococci from children with lobar pneumonia.

Contamination with saliva from the mouth seemed not to interfere in the determination of the type of the causative pneumococcus.

STUDIES ON RESPIRATORY DISEASES

XIX. UNTREATED BILE AS A SOLVENT FOR PNEUMOCOCCI *

FRANK B. KELLY AND HARRY GUSSIN

From the Department of Hygiene and Bacteriology, University of Chicago

Bile solubility is now generally recognized by laboratory workers as an important criterion for differentiating the pneumococci from green-producing streptococci. Levy,¹ Lyall,² Cole³ and his co-workers, and Aschner⁴ have maintained that considerable reliance can be placed on this test. Less favorable opinions have also been given. Neufeld,⁵ in his article describing the discovery of the phenomenon, stated that some strains of pneumococci were not very soluble. Schultz⁶ claimed that some streptococci were dissolved, and that pneumococci were variable in solubility. As recently as 1918, Keegan,⁷ Nuzum⁸ and Dunn⁹ have considered the test unreliable. Mair¹⁰ has taken a conservative attitude, stating that all workers agree that all bile-soluble cocci are pneumococci, but that not all pneumococci are bile soluble.

A contributing factor to this variation of opinion has been the lack of uniformity in making the tests. As originally performed by Neufeld,⁵ solubility was determined with fresh, whole bile. Both Neufeld⁵ and Mandlebaum¹¹ found bile-salt preparations inferior. Levy,¹ on the other hand, used only solutions of sodium taurocholate. Nicolle and Adil Bey¹² reported that the bacteriolytic effect of whole bile was not weakened by heating to 115 C., and considered that a 1:1,000 solution of sodium choleate was equivalent in bacteriolytic

* This is one of a series of studies carried out in connection with the Influenza Commission established and financially aided by the Metropolitan Life Insurance Company of New York. Part of the expense of these studies has been met by a grant from the University of Chicago.

Received for publication, May 28, 1924.

¹ Virchows Arch. f. path. Anat., 1907, 187, p. 327.

² Jour. Med. Res., 1917, 30, pp. 487 and 515.

³ Jour. Exper. Med., 1912, 16, p. 644.

⁴ Jour. Infect. Dis., 1917, 21, p. 409.

⁵ Ztschr. f. Hyg. u. Infektionskr., 1900, 34, p. 454.

⁶ München. med. Wchnschr., 1907, 54, p. 1431.

⁷ Jour. Am. Med. Assn., 1918, 71, p. 1051.

⁸ Ibid., p. 1562.

⁹ Ibid., p. 2128.

¹⁰ Jour. Path. & Bacteriol., 1917, 21, p. 305.

¹¹ München. med. Wchnschr., 1907, 54, p. 1431.

¹² Ann. de l'Inst. Pasteur, 1907, 21, p. 20.

ability to a 1:10 dilution of whole bile. Grixoni's¹³ results showed fresh, whole bile to be more effective than bile that had been heated or sterilized by any other process, such as the addition of a preservative. Mair obtained excellent results with a 1:1,000 dilution of sodium desoxycholate, although impurities in the solution frequently produced a confusing opalescence.

Libman and Rosenthal,¹⁴ in the initial work in this country on bile solubility, apparently used fresh, whole bile. Cole,³ in 1914, chose bile-salt solutions. The authors of Monograph No. 7 (1917)¹⁵ of the Rockefeller Institute advocated autoclaved whole bile. Since these workers were the pioneers in typing pneumococci, their methods have been widely copied throughout the country, and autoclaved bile has probably been more generally used for the bile-solubility test than any other bile preparation. We have found no reports of the use of untreated bile as a routine solvent since 1909.

The kind of medium employed for growth has likewise been variable. Neufeld, Mandelbaum, and Levy, in their initial observations, used plain beef broth cultures. Nicolle and Adil Bey used dextrose broth and ascitic-fluid broth, and reported that the cultures in ascitic-fluid broth did not dissolve as easily as those in plain broth. Dextrose broth precipitated the sodium choleate because of the acidity of the culture. Adding alkali had no effect on the organisms, which were found refractory to dissolution because of the presence of "hindering substances." The latter were removed by washing the cultures in physiologic salt solution, centrifuging, and restoring them to their original volume, after which the cocci were dissolved. This report has apparently been the basis of the ban on dextrose broth as a medium for growing pneumococci for the bile-solubility test. Libman and Rosenthal cited Nicolle and Adil Bey's work, although they reported solution of 5 strains of pneumococci grown in dextrose broth. Libman and Rosenthal seem to have used fresh, whole bile. Cole stated that even the washing of plain broth cultures in dextrose solution made them less readily soluble; he used bile-salt preparations only. Aschner was so impressed with the belief that dextrose broth interfered with the bile solubility, that when he failed to secure sufficient growth in plain broth to carry out the test, he did not try dextrose broth, men-

¹³ Rev. Crit. Clin. Med., 1909, 10, p. 17.

¹⁴ Proc. N. Y. Path. Soc., 1908, 8, p. 40.

¹⁵ P. 20.

tioning Nicolle and Adil Bey's results as his reason. Sellards, and Lord and Nye used dextrose meat-infusion broth cultures and obtained lysis. They made no reference to the reported undesirability of dextrose.

This confusion concerning the bile-solubility test is reflected in the textbooks on bacteriology. Examination of any group of them, such as Jordan,¹⁶ Stitt,¹⁷ Hiss and Zinsser,¹⁸ and Park and Williams¹⁹ confirms the existence of marked variation in the method of conducting the test.

While it is an apparently simple matter to make a bile-solubility test, in practice it has been difficult to interpret the appearance of the organism-bile mixture. The scant growth obtained in plain broth has afforded very thin emulsions; the acid developed in sugar broths has precipitated autoclaved whole bile and bile salt solutions; and the opalescence in heated bile has rendered determination of complete solubility quite impossible.

We have found that the use of whole, untreated ox bile permits the employment of sugar broths which afford emulsions sufficiently dense to make the readings of solubility a simple and accurate procedure. This fact, while seemingly at variance with results and contentions of other workers, particularly Nicolle and Adil Bey's, is not so actually.

The discrepancy results from the misinterpretation of the work of Nicolle and Adil Bey. If their paper is carefully read, it appears that after reporting the solvent effect of whole bile on plain broth cultures they say, "Then we turn to a product which is impure but can be readily used, the sodium choleate of the Pharmacopée Germanique. This product, which is very soluble, when added to the cultures in the proportion of 1:1,000 also dissolves them readily, even as well as beef bile in the ratio of 1:10. . . . The cultures in Bouillon-Martin-ascite ($\frac{1}{3}$ ascitic fluid) are less soluble than cultures in Bouillon-Martin (plain broth); with 1:1,000 choleate, one gets nothing but an incomplete bacteriolysis. . . . The cultures in dextrose broth (plain broth, 2% peptone, and 0.5% dextrose) precipitate the choleate on account of the strong acidity." This paragraph is their only reference to the solubility of dextrose broth cultures. They confined their subsequent observations entirely to the bile-salt preparations and drew conclusions

¹⁶ General Bacteriology, 1922, p. 226.

¹⁷ Practical Bacteriology, Blood Work, Parasitology, 1923, p. 94.

¹⁸ Textbook of Bacteriology, 1922, p. 453.

¹⁹ Pathogenic Microorganism, 1917, p. 269.

from their results with it. They do not again mention the use of whole bile and do not even imply that they tried the solution of dextrose broth cultures with whole bile.

There is no doubt that untreated bile dissolves pneumococci better than the bile preparations ordinarily used for this purpose. Whole ox bile which has never been heated or sterilized by any means is superior to the autoclaved or Arnoldized product, or to the common bile-salt solutions. Its solutions are clearer, it can be used with ordinary sugar broths, and it can be kept indefinitely without loss of its solvent property.

An opalescence results from heating bile to 100 C., and this becomes greater after autoclaving. This opalescence is confusing in making final readings of solubilities, even if a control tube is used to which bile is added just previous to making a reading. There is no opalescence in untreated bile even after storage for several weeks, and no uncertainty in the final results, for there is complete transparency of the solutions of the organisms.

Dextrose broth gives luxuriant growths of pneumococci but, as shown above, has been described as unsuitable on the ground that the acidity developed in it makes the organisms less soluble and also precipitates the bile preparations. Plain broth cultures have been advocated as necessary for the test, even though admitted to be unsatisfactory because of scant growth. It is true, however, that untreated bile is not precipitated by the highest acidity that develops in dextrose broth cultures of pneumococci, and that it can dissolve completely an unwashed emulsion of the organisms at least 10 times as dense as the 24-hour growth in such broth. Freshly collected ox bile commonly is golden brown. The bile can be kept on ice or at room temperature. A slimy scum forms on its surface and a heavy precipitate accumulates. A clear golden brown liquid gathers in the center of the container. When the bile is to be used, a pipet is passed through the surface scum until the tip of the pipet projects well into the clear fluid. The bile is drawn into the pipet, the pipet removed and wiped clean. The bile is used in a proportion of 1:2 of the volume of the emulsion of organisms. It dissolves the pneumococci within 20 minutes.

For this comparative study we used 24-hour glucose broth cultures of 41 strains of pneumococci, including ten strains of type 1, eight of type 2, five of type 3, and eighteen of type 4. These strains had been isolated from cases of lobar pneumonia at various times during

the preceding 10 months. They were grown in 500 c.c. flasks, centrifuged, and taken up in normal salt solution; they were not washed. The emulsions were made of a degree of turbidity such that when placed in a tube of 1.5 cm. outside diameter, the outline of an inoculating needle held tightly against the opposite side of the tube was indistinct. All the tubes used in this test were 1.5 cm. outside diameter, 1.2 cm. inside. The ratio of bile to emulsion was 1:2. A 10% solution of sodium taurocholate, autoclaved, whole bile, and untreated, whole bile which had been kept on ice 2½ months, were compared. The control tubes had normal salt solution added to the emulsion to make their volumes equivalent to the volumes in the tubes containing bile. The tubes were incubated in a water bath at 42 C. for 20 minutes, and the readings made either by holding the tubes about two feet from a lighted, white-frosted, electric bulb; or by using the clear sky as a background. Only transmitted light was used.

Almost immediately on adding the sodium taurocholate a dense light colored precipitate was formed, rendering an exact determination of solution impossible. Most of this opacity cleared on the addition of from 5 to 15 drops of N/10 sodium hydroxide solution. Designating + + + + as complete solution, the sodium taurocholate gave final readings of + + and + + +, autoclaved bile, + + +; and stored, untreated bile, + + + +. An opalescence made the autoclaved bile appear slightly cloudy, a confusing sheen persisting even after the addition of sodium hydroxide, giving the appearance of incomplete solution.

The acidity of dextrose broth cultures of pneumococci after 24 hours' incubation at 37 C., is, under ordinary conditions, P_H 5.5-5.0. We have determined this frequently by both the colorimetric and potentiometer methods. It requires an initial concentration of but 0.4% of the dextrose to produce this acidity. Plate inoculations have shown such cultures still viable. We have confirmed this several times, bearing in mind the report²⁰ that a few hours' exposure at P_H 5.1 kills pneumococci and that the dead organisms will not dissolve in bile. However, Avery and Cullen²¹ have recently reported solution of killed pneumococci by an intracellular enzyme. In order to be certain that our observations were being made in suspensions of the highest acidity that could be developed by the dextrose broth culture, we took up

²⁰ Lord, F. T.: Jour. Exper. Med., 1919, 30, p. 389.

²¹ Avery and Cullen: Ibid., 1923, 38, p. 199.

centrifuged organisms in a buffer solution of P_H 4.5 and repeated the foregoing observations with the same bile preparations. The results were practically identical with those obtained with emulsions in normal salt solution. The untreated bile gave complete solution.

To determine the acidity that would precipitate these three bile preparations, various quantities of different concentrations of hydrochloric acid were added to equal amounts of bile. The results, together with the approximate P_H readings are shown in table 1. It would be

TABLE 1
EFFECT OF ACID BILE

HCl P_H Value	Amount Required to Produce Precipitate in 2 C c. Bile Preparation		
	Fresh Bile	Autoclaved Bile	Sodium Taurocholate
P_H 5	No precipitate even with 5 c c. of acid	Slight cloudiness with 2.5 c c.; not permanent even with 5 c c.	Remains cloudy after adding 2.5 c c. acid
P_H 4	No precipitate even with 5 c c. of acid	Slight cloudiness with 1 c c.; not permanent with 5 c c.	Remains cloudy after adding 2.5 c c. acid
P_H 3	Begins to appear with 1.5 c c. acid, disappearing on shaking; permanent with 3.4 c c.	Cloudiness with 1 c c.; permanent with 3.4 c c.	Remains cloudy with 1 c c.
P_H 2	Begins with 0.25 c c. acid; remains with 0.5 c c.	Begins with and remains with 0.2 c c.	Remains cloudy with 0.03 c c.
P_H 1	Begins and remains with 0.05 c c.	Begins and remains with 0.05 c c.	Remains cloudy with smallest drop that could be added

necessary for a dextrose broth culture to reach an acidity of P_H 3 before any precipitation could occur with stored untreated bile. This figure is never attained.

Quantitative values of the solvent properties of the 3 bile preparations were sought in 11 of the emulsions selected at random. Stored, untreated bile in a ratio of 1:4 completely dissolved them all. The autoclaved product was similarly effective in only 2 cases, and in these required a 1:2 ratio of bile. A precipitate immediately appeared with the sodium taurocholate, preventing observation of the extent of solution until alkali was added. It was found necessary to use not less than 0.4 c c. of 4/10 Na OH to 2:0 c c. of emulsion before clearing occurred. In no case did complete solution take place in a proportion of 1:10 as it did in some cases with stored, untreated bile.

SUMMARY

Stored, untreated, whole ox bile dissolves pneumococci better than the bile preparations ordinarily used. It dissolves dextrose broth cultures readily with no interference from precipitation. Misinterpretation of Nicolle and Adil Bey's reports has been a factor in the failure to recognize these points.

EFFECT OF SUPRARENALECTOMY IN RATS ON AGGLUTININ FORMATION

HENRY L. JAFFE AND DAVID MARINE

From the Division of Laboratories, Montefiore Hospital, New York

Také and Marine¹ recently reported that rabbits with high grade but sublethal suprarenal insufficiency gave hemolysin titers averaging more than twice as high as the controls. We subsequently showed that suprarenalectomized rats produce higher agglutinin titers than control rats.² The purpose of this communication is to report in greater detail further studies on suprarenalectomized rats. The literature was reviewed in the previous papers, and no additional studies dealing specifically with this point have appeared since.

METHODS

We used young tame albino rats (*Mus norvegicus albinus*) reared and interbred in the laboratory. The operative technic was standardized; both glands were removed through the dorsal route at one sitting. Ether anesthesia was always used by the open method. Food was withheld for 18 hours before operation and for 12 hours after operation. For two days following suprarenal ablation, bread and milk only were given, then a dry food mixture and vegetables were added. The operated rats were kept for two weeks in individual cages, care being taken that they were warm; they were then placed in larger cages with the nonoperated controls. During the course of these experiments the controls and operated rats were fed exactly alike. Generally, the control and operated rats were litter mates of the same sex, and of approximately the same weight. Necropsies were made on all rats, and a careful search made for accessory suprarenal tissue.

EXPERIMENTS

Our studies include 30 rats which were doubly suprarenalectomized, and which survived the injections of standard typhoid vaccine; 3 which were unilaterally suprarenalectomized; 4 in which the fat about the glands was torn; and 31 nonoperated controls. All animals were young

Received for publication, May 31, 1924.

¹ Jour. Infect. Dis., 1923, 33, p. 217.

² Jaffe, H. L., and Marine, D.: Proc. Soc. Exper. Biol. & Med., 1923, 21, p. 64.

adults at the time of immunization. The operated rats were immunized between 8 and 40 days after suprarenalectomy, and no animals were used unless they were alert, active, eating, and not losing appreciably in weight.

Standard typhoid vaccine containing 1 billion bacilli per cubic centimeter was used for immunization. The rats were injected intraperitoneally every third day and were given 3 or 4 doses. The operated and control rats were divided into 5 series and received initial doses of vaccine varying from 0.2 to 0.5 c.c., and final doses varying from 0.2 to 1.0 c.c. The amount of vaccine injected depended on the duration of time between suprarenalectomy and immunization, since recently suprarenalectomized rats show a marked lowering of resistance to typhoid vaccine; but suprarenalectomized and control rats in each group always received the same amounts of vaccine.

Natural typhoid agglutinin was determined in a number of the rats before the injections were begun, and in no instance was there any agglutination in dilutions greater than 1:20. The titrations were made every 3rd or 4th day after the last injection, the animals being bled from the tail, and the serums of both the operated and control rats were examined on the same day. In all titrations, we used a formalinized stock culture of an old laboratory strain of typhoid bacillus made up according to the method of Dreyer. The titrations were made with 1 c.c. of diluted serum and 1 c.c. of typhoid emulsion, the tubes being placed in a water bath at 37 C. for 2½ hours, and then in the icebox over night. The results were read the next morning. In the table we present for comparison the averaged titers developed by each group of suprarenalectomized rats and their controls.

In the first series, 14 doubly suprarenalectomized and 15 control rats received from 0.6 to 0.8 c.c. of vaccine in the 3 injections, the operated rats being immunized from 8 to 13 days after bilateral suprarenal ablation. The averaged titers developed by these 14 suprarenalectomized rats were 820, 910, and 725 for the 1st, 2nd, and 3rd titrations, while the control rats developed titers of 395, 305, and 275 (see table, series I). Four of these 14 suprarenalectomized rats died before the titrations were completed, but blood for at least one titration was obtained from each. Postmortem examination of these rats revealed nothing striking; the thymus was small in 2 animals and large in 2 others. Two rats, killed 76 and 203 days, respectively, after operation, showed unilaterally small, regenerated suprarenal masses, but both

developed high titers when immunized 13 days after operation. Two other suprarenalectomized rats, immunized 11 and 13 days after operation, did not develop higher titers than their controls. They did not react severely to the injections of vaccine, and at necropsy no macroscopic accessories were found. In general, the recently suprarenalectomized rats which reacted severely to the injections, becoming dull and weak, responded with the highest titers. The titers developed by the 14 suprarenalectomized rats in this series were from 2 to 3 times as high as those by their controls.

In the second series, 7 suprarenalectomized rats and 5 controls were immunized with from 2 to 2.5 c.c. of vaccine, the operated animals being injected on the 16th and 17th day after bilateral suprarenal ablation (see table, series 2). The suprarenalectomized rats developed titers of 975, 950, and 500 for the 1st, 2nd, and 3rd titrations, while their controls showed titers of 700, 435, and 300. The antibody response elicited in these animals 16 to 17 days after suprarenalectomy is almost twice that of the controls, but the agglutinin response in our rats did not vary proportionately with the amount of antigen injected.

In the third series, 4 suprarenalectomized rats were immunized 25 days after operation, together with 4 controls. Each rat received 3 injections of 0.5 c.c. of vaccine (see table, series 3). The titers developed in the operated rats were only slightly higher than those developed in the controls.

The fourth series consisted of 4 operated rats, together with 4 controls, immunized 40 days after suprarenalectomy (see table, series 4). Each rat received three injections of 1 c.c. of vaccine. The agglutinin titers developed in both groups were substantially the same.

A fifth series of experiments was carried out to study the difference between unilaterally and bilaterally suprarenalectomized rats. Two bilaterally and 3 unilaterally suprarenalectomized rats were immunized 17 days after operation, receiving a total of 0.6 c.c. of vaccine in the 3 injections. The agglutinin titers developed in the bilaterally suprarenalectomized rats were somewhat higher than those developed in the unilaterally suprarenalectomized animals. However, the agglutinin response at 17 days to 0.6 c.c. of vaccine was less than the response to the same amount of vaccine from 8 to 13 days after suprarenalectomy.

Control experiments to evaluate the factor of operation were carried out; 4 rats in which the fat about the suprarenal glands was

torn were immunized 13 days after operation. Each rat received 3 injections of 0.2 c c. of vaccine. The agglutinin titers were practically the same as those developed by the 15 control rats in series I, receiving the same amount of vaccine. This experiment would seem to indicate that the difference in agglutinin response between suprarenalectomized and control rats was not dependent on operative trauma.

TABLE 1
AGGLUTININ FORMALIN AFTER SUPRARENALECTOMY

Series	No. of Rats	Days After Suprarenal-ectomy	Amount Vaccine Every 3d Day, C c.	Average Titers		
				1	2	3
1	1	8	0.25, 0.25, 0.25	1,000	1,200	800
	8	Control	300	300	300
	4	11	0.2, 0.2, 0.4	675	675	600
	4	Control	525	400	350
	5	13	0.2, 0.2, 0.2	760	870	800
	5	Control	360	220	200
2	4	13	0.2, 0.2, 0.2	1,000	900	700
	4	Control	400	300	250
	4	16	0.3, 0.5, 0.75	1,150	900	500
	2	Control	800	400	300
	2	17	0.5, 0.5, 1.0	800	1,000	500
	3	Control	600	470	300
3	4	25	0.5, 0.5, 0.5	600	600	500
	4	Control	550	400	400
4	4	40	1.0, 1.0, 1.0	900	650	400
	4	Control	1,000	600	500
5	2	17	0.02, 0.02, 0.02	500	500	450
	3	Unilateral suprarenal ectomy	500	330	270

DISCUSSION

It has been shown that rats surviving, in good condition, removal of both suprarenal glands develop higher agglutinin titers than their controls if immunized within 3 weeks after operation. The most striking difference in response is obtained when the rats are immunized with small amounts of vaccine during the second week after suprarenal-ectomy. They then develop titers from 2 to 3 times as high as their controls. After the 3rd week, the difference in the agglutinin response between immunized suprarenalectomized and control rats begins to diminish, and after the 6th week we were unable to demonstrate any difference in this respect between the control and suprarenalectomized rats.

The difference in the agglutinin response between recently suprarenalectomized and control rats parallels and is closely related to the diminution of the resistance of these animals to vaccine. Numerous investigators have studied the changes in the resistance of surviving suprarenalectomized animals; and the recent comprehensive and adequately controlled work of Lewis³ and of Scott⁴ has shown that rats shortly after suprarenalectomy are highly susceptible to small doses of a large variety of drugs and toxins, and that resistance to these poisons is reestablished to a considerable degree in about 6 weeks.

In this work, we have had the opportunity to collect data relevant to the compensation and reestablishment of the resistance of suprarenalectomized rats as measured by susceptibility to intraperitoneal injections of typhoid vaccine. We found that 8 days after suprarenalectomy, 75% of our rats succumbed to an injection of 0.25 c.c. of vaccine, and from 11 to 13 days after operation, 43% succumbed to 0.2 c.c. of vaccine. On the other hand, 0.3 c.c. of vaccine was not lethal from 16 to 17 days after suprarenalectomy, but 0.5 c.c. at this time killed 2 out of 5 rats (40%). The injection of 1 c.c. of vaccine 23 days after suprarenalectomy killed 33% of a small series of rats; while 2 c.c. at 31 days was not lethal, but 4 c.c. killed 2 rats which were injected at this time. Forty-three days after suprarenalectomy, 2 rats were killed by 4 c.c. of vaccine, and at 51 days 2 out of 6 rats survived this amount; while 61 days after operation 2 out of 4 survived 5 c.c. of vaccine. Normal adult rats readily survive injections of 5 c.c. standard typhoid vaccine.

No further data in explanation of the increase in agglutinin formation shortly after suprarenalectomy over that offered by Také and Marine have been developed beyond the facts that in the rat the antibody response is greatest when the degree of suprarenal insufficiency is greatest as measured by the resistance of the animal to injection of typhoid vaccine, and that as the animal recovers from suprarenal insufficiency the antibody response falls. In our series, this fall reached the normal level between the 30th and 50th day after suprarenalectomy. This would seem to indicate that the antigenic effect of a given dose of vaccine is many times greater in recently suprarenalectomized animals than in nonoperated controls, or in suprarenalectomized rats which have been allowed to go for 6 or more weeks before immunization.

³ Am. Jour. Physiol., 1923, 64, p. 506.

⁴ Jour. Exper. Med., 1923, 38, p. 543.

In the previously reported work by Také and Marine on hemolysin formation in the rabbit, no noteworthy difference in antibody formation was observed that might be related to the time interval between suprarenalectomy and immunization, nor was the resistance to the injection of typhoid vaccine appreciably reestablished as late as 5 months after bilateral suprarenalectomy. Rabbits were killed by the intravenous injection of as little as 0.2 c.c. of standard typhoid vaccine. These differences between the rabbit and the rat are due, we believe, to the fact that suprarenalectomy in the rabbit produces a more profound and prolonged insufficiency not so completely compensated for by the presence of accessory inter-renal tissue, or by the rearrangements in the functional inter-relations of other glands which follow extirpation of the suprarenals. While qualitatively the functions of the suprarenals are probably the same down the animal scale even to the cartilaginous fishes, quantitatively the suprarenal glands probably stand in different relationships to the functional needs of the organism in the different species. This also explains to some degree the inability of some animals such as the dog and guinea-pig to survive removal of both main glands. Other factors, as was previously pointed out, may come into play, as for instance the lymphatic and hematopoietic tissues, which have been shown to undergo hyperplasia following suprarenalectomy, and while all body cells take part in antibody formation, it appears to be established that these tissues are particularly concerned. Or again, the heightened antibody formation may be another manifestation of the general increase in tissue reactivity and irritability which follows sublethal by sufficient injury to the inter-renal glands, and which is also shown by the increased metabolism in rabbits, cats, and dogs, and by the increased sexual activity.⁵

There is no doubt that changes in serum concentration markedly affect antibody titers. This possibility must be considered when working with suprarenalectomized animals, and while we have a great deal of negative evidence obtained from blood counts and blood chemistry, nevertheless this factor is not absolutely eliminated.

CONCLUSIONS

Rats immunized with typhoid vaccine within 3 weeks following bilateral suprarenalectomy, had agglutinin titers averaging 2 to 3 times greater than their controls.

⁵ Marine, D., and Baumann, E. J.: *Am. Jour. Physiol.*, 1921, 57, p. 135.

Six weeks after suprarenalectomy no difference in the agglutinin response between normal and suprarenalectomized rats surviving the operation in good condition could be determined, although their resistance to large doses of vaccine was still somewhat below that of normals.

There is a marked lowering of the resistance of recently suprarenalectomized rats to typhoid vaccine, which is rapidly compensated for, so that some rats 9 weeks after bilateral suprarenalectomy may withstand as much as 5 c c. of typhoid vaccine intraperitoneally.

It is suggested that the increased antibody formation in recently suprarenalectomized rats is intimately related to their decreased resistance.

INVESTIGATIONS ON BACTERIAL HYBRIDS

ONE PLATE

ERNST ALMQUIST

Stockholm, Sweden

In another paper,¹ I presented a short review of the life cycles of some pathogenic bacteria and pointed out that our knowledge of this subject is incomplete. I suggested that especially the study of the symplastic stage might lead to unexpected results. I thought in the first place of facts indicating the occurrence of sexual processes in the development of the bacteria.

THE STARTING POINT OF MY RESEARCHES

In plating a strain of *B. dysenteriae* "Shiga," received in 1915 from the Berlin Gesundheitsamt, I discovered that the same colonies may contain both slender and big rods. Two different forms could be separated, one broad, short, and immotile, another motile and almost similar to *B. typhosus*. I cultivated these strains for several years under different conditions, at low temperature, on dry substrates, etc. Apparently both remained constant; 1 mg. of each was sufficient to kill a guinea-pig.

Koraen inoculated rabbits with both strains separately, and made the interesting observation that each serum agglutinated not only its own strain, but also the other, and in addition several different cultures of *B. dysenteriae* at least up to 1:2,000.

In order to secure more information in this direction, I grew mixed cultures of different species. Many tests gave negative results, e. g., *Sp. cholerae* or *B. paratyphosus* together with *B. typhosus* or *B. dysenteriae*, also some strains of *B. dysenteriae*, labeled "Shiga" when cultivated with *B. typhosus*. Other strains of *B. dysenteriae*, however, produced unusual forms in combination with *B. typhosus*.

The methods for growing mixed cultures have been much varied. Usually a dry substrate at 10-14 C., but sometimes at 18 C., was used. By repeated plating I tried to find new, different individuals. I

Received for publication, June 2, 1924.

¹ Variation and Life Cycles of Pathogenic Bacteria, *J. Infect. Dis.*, 1922, 31, p. 483.

examined their motility, agglutination, life cycle, etc. All strains used for mixed cultures were first purified by plating. The agglutination was always controlled by the salt water test.

MIXED CULTURES OF MINUTE FORMS

In my earlier paper,² I discussed the possibility of stimulating bacterial mutation by filtering, plating, and heating cultures of *B. typhosus*. Different small organisms could be isolated. A minute form, cultivated in 1916 from typhosus rods which had been grown on algae, propagates slowly on agar as a yellow layer and consists of thin curved needles. Soon after another strain was isolated from the bladder of a sick man. It grows on agar as a mucous membrane composed of curved needles, frequently producing small or larger granules. Both strains are rather similar, scarcely motile, but the latter has longer needles in the form of very fine threads. Both are apparently constant. For about 6 months I cultivated them together on agar at about 18 C., and examined their behavior by frequently repeated plating.

Evidently, the two original strains have produced quite different forms; only one of them may be mentioned here. Among the yellow colonies of the original strains, frequently larger limpid colonies were found, 1 cm. in diameter. These limpid colonies always produced small yellow points and contained cells similar to those of the parent strains, but also rods of the size of *B. typhosus*, which were motile and rather protoplasmatic.

MIXED CULTURES OF *B. TYPHOSUS* AND *B. DYSENTERIAE*

One strain of *B. dysenteriae* "Shiga," received from Berlin (Robert Koch Institute), produced interesting individuals when grown together with *B. typhosus*. A few of the results obtained in 1916-1918 may be described. The parent strains grew as big rods and threads.

A mixed culture on dry agar kept at 18 C. for 2 months contained motile individuals which were agglutinated both by typhosus and dysenteriae serum (1:500). On the plate, this strain furnished growth of different kinds. Among 10 colonies, 7 were agglutinated by typhosus serum only, but 3 were agglutinated by both serums (1:500). These 3 colonies were replated. Most of the resulting colonies were agglutinated by typhosus, a few by dysenteriae serum, and only one strain by both serums (1:500). This culture was studied for a long time; the

² Ibid., p. 487.

agglutinability diminished gradually, and not a few subcultures were quite inagglutinable.

A second mixed culture of the same parent strains grew on dry agar in a similar manner. Within two weeks, plating showed the presence of some motile rods that were strongly agglutinated both by typhosus and dysenteriae serum (1:100). Colonies of big motile inagglutinable rods also appeared. One colony was found to contain long motile threads, agglutinable only by dysenteriae serum; later its offspring became inagglutinable, or was agglutinated only by typhosus serum.

Several other mixed cultures of the same parent strains gave similar results when grown on serum or on potato at different temperatures. The colonies obtained by plating were inagglutinable, or agglutinable either by one serum or by both serums.

Thus all mixed cultures furnished motile rods which were agglutinated rather strongly by both serums. But the offspring did not show constancy. The agglutinability by dysenteriae serum usually disappears first, while the action of typhosus serum is more persistent.

We are not yet able to explain these observations, but they seem to indicate a segregation of hybrid strains, hybrids of *B. typhosus* and *B. dysenteriae*. The variable behavior in the agglutination test agrees well with this hypothesis; and further observations prove, in fact, the existence of hybrids from the parent strains named above.

FURTHER EXPERIMENTS WITH MIXED CULTURES

In 1920, fresh cultures of *B. dysenteriae* were received from the Breslau Hygienic Laboratory. One strain, labeled "Shiga," showed big rods and was strongly agglutinated by a serum labeled "Kruse." It did not produce new forms when mixed with *B. typhosus*. Two other strains, labeled "Y" and "Flexner," were agglutinated only by a serum labeled "pseudodysentery." The strain Y contained big, short rods, often fusiform to rhombic, the latter big rods, with a tendency to form threads; both were scarcely toxic.

(1) Experiments with *B. typhosus* and *B. dysenteriae* Y. Mixed cultures were grown on dry agar at 12 C. When plated after 1 month, colonies of motile rods were found either similar to *B. typhosus*, or of broader type. The layers on agar were not quite smooth, and they soon became dotted; small secondary colonies frequently appeared. Some of them were strongly agglutinated by both typhosus and dysenteriae serum, others by one serum only.

Single-cell cultures were made from this strain. The pure culture obtained agreed with the description given above. The rods were motile; slender and big rods appeared simultaneously; sometimes long threads were found. At low temperature, numerous globular cells and some large dividing ovals were present. The agglutinability was strong in typhosus, less so in dysenteriae serum (1:100). A guinea-pig was killed by 2 mg. The life cycle of this culture seems to differ from that of the parent strains.

(2) Mixed cultures of typhosus rods and of the dysenteriae strain Flexner produced forms that were different from the offspring of Y, described above. The new strains were very much inclined to produce minute forms which were easily separated by plating. Sometimes they appeared in the agar tubes as extended yellow surface growths; in other cases the agar culture showed a dotted appearance. The rods were motile, morphologically similar to *B. typhosus*; at 12 C. they produced many germinating globules. They were strongly agglutinable by typhosus, sometimes also by dysenteriae serum.

A mixed culture made in February, 1923, produced after 1 month's cultivation on agar at 12 C. small yellow colonies on the plate. After 1 year, two rods were isolated; both single cell cultures agree with the description given above. The rod is motile and rather similar to *B. typhosus*. Big rods (up to 1μ in width) are also produced, as well as many threads, budding globules, large ovals, and an immense number of grains or fine needles. Agglutination is strong with typhosus, weak with dysenteriae, serum; 2 mg. killed a guinea-pig. The tendency to produce minute forms is marked with these strains.

Another mixed culture made in February, 1922, was also plated after 1 month at 12 C. Minute forms soon appeared, partly as small yellow points on the large colonies, partly as small yellow colonies within the agar, partly as dotted layers on the surface. After a year's cultivation, 3 rods were isolated. These single cell cultures again behave exactly like those mentioned before. Agglutinability and toxicity are the same.

The main character of these strains appears after 1 or 2 days' cultivation at 35 C. The slender rods then transform themselves to big shapeless rods, large ovals, irregular quadrangles, $1-2 \times 2$ or $2 \times 3\mu$, all actively motile, dividing by fission, and producing bright grains which are easily stained. When transferred to 18 C., this culture soon reproduces the regular big rods.

Most of the globular forms of *B. typhosus* were not motile. They never propagated by fission, but germinated by budding, and they produced within the cells minute forms which were difficult to observe. Few individuals resembled those of the hybrid form, scarcely any in the cultures kept at 35 C.

The other Flexner strain is quite immotile, inagglutinable by typhosus serum, and scarcely toxic. It produces only a small number of irregular cells.

For the hybrid form I propose the name *B. diploides*, because the diploid forms are of considerable importance in the cultures. It exhibits characters of both parent strains in quite the same manner as is done by other hybrids.

Figure *F* of plate 1 illustrates the big rods, figs. *D* and *G* the irregular forms grown at 35 C., both budding and dividing. Figure *E* was made from a slide stained after Heidenhain, demonstrating the nuclei.

STUDIES ON BACTERIAL NUCLEI

Investigations on the bacterial nuclei are necessary in order to solve the problem of bacterial sexuality. It is especially important to look for the haploid nuclei and for their copulation to diploid ones. In fact, I have been successful in staining them by Heidenhain's method. A priori I relied on their visibility in the symplastic stage and in all minute forms; many of both proved, indeed, fit for staining, as has been described elsewhere.³

In the large globes and other big forms developing from the symplastic stage, I found large nuclei, 1μ in diameter; in the minute forms, however, there were very small nuclei, similar to bright points, often in rows. The former nucleus is diploid, the latter haploid. The diploid nuclei must be reduced for reproducing the small kind.

The results of these observations are almost similar to those secured by Jahn in 1911 concerning the myxomycet *Bodhamia*, whose nuclear changes are quite analogous to those of *Sp. cholerae*. In the ameba stage, the nuclei are haploid, those of the plasmodium are diploid. Reduction takes place before the spores are produced, and the spore germinates to an ameba. Low temperature (8-18 C.) is necessary for the development of the plasmodium. The nuclei in the small curved cells of *Sp. cholerae* are likewise haploid. At low temperature, within

³ Studien über die Sexualität pathogener Bakterien, Ztschr. f. Hyg. u. Infektionskrankh., 1923, 101, p. 15.

a few days, thick diploid rods and diploid globes appear ; the copulation is then past. The great globes germinate again to fine haploid spirilla after their nuclei have been reduced.

Figure *A* of plate 1 illustrates the globular forms of *Sp. cholerae*, grown at low temperature; fig. *B* shows their nuclei: three haploid nuclei in a spirillum, and apparently free diploid nuclei. Figure *C* was made from *B. typhosus*: a long row of haploid nuclei in a rod, and apparently free diploid nuclei. Figure *E* shows diploid nuclei of *B. diploides* and haploid ones in a bud and in a fine rod.

CONCLUSIONS

Bacterial sexuality is proved by the discovery of *B. diploides*, a hybrid of *B. typhosus* and *B. dysenteriae*. The existence of haploid and diploid nuclei in bacteria also indicates sexuality.

Bacterial sexuality and the existence of bacterial hybrids are of importance both to bacteriology and to epidemiology.

PLATE I

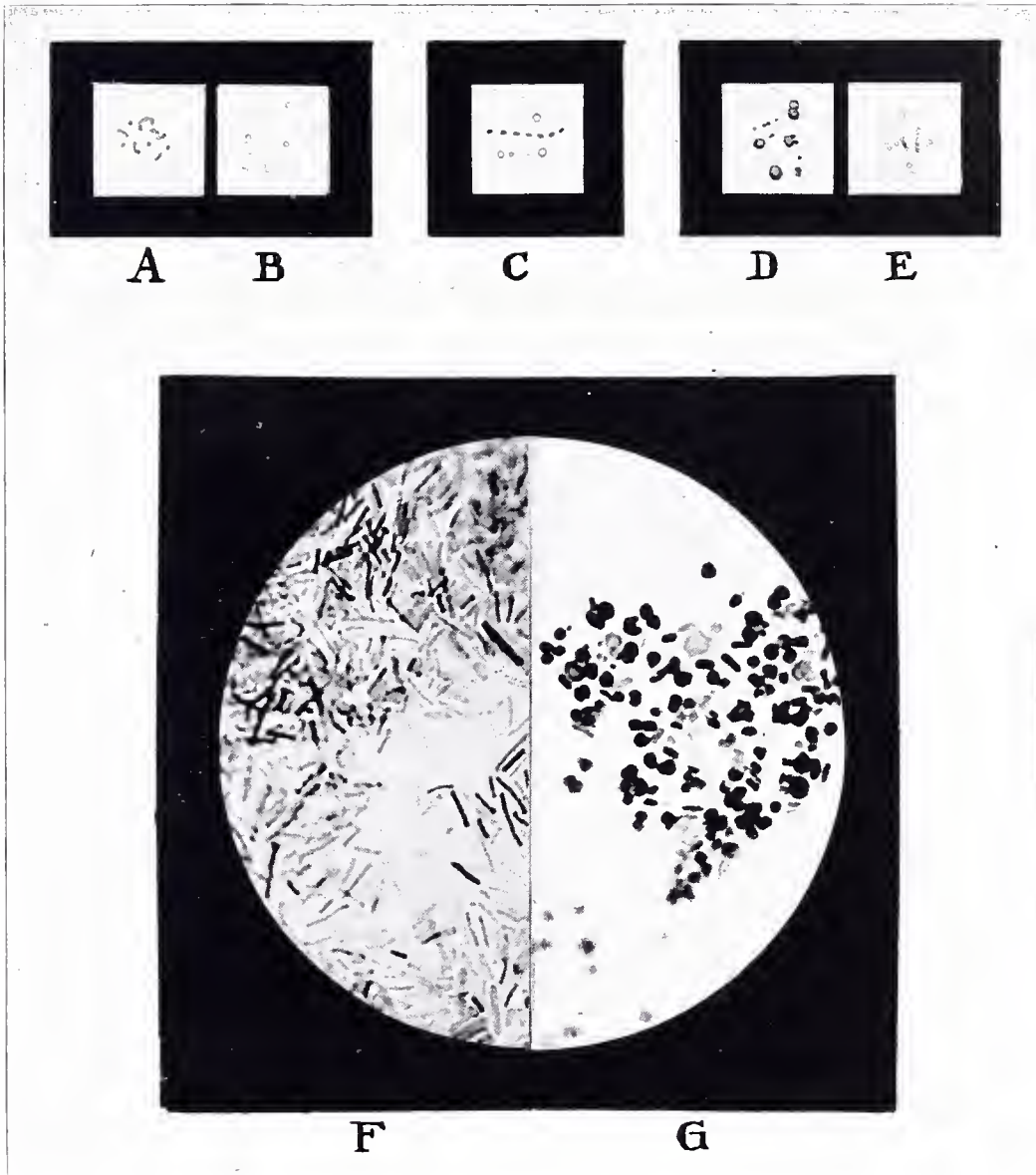


Plate 1.—The drawings A to E were made directly from the microscopic observation, $\times 1000$. Figures F and G are photomicrographs, $\times 1280$. A, *Spirillum cholerae*, 4 days on agar at 15 C., stained with fuchsin; B, *Spirillum cholerae*, 3 weeks on agar at 12 C., Heidenhain stain; C, *B. typhosus*, 3 weeks on agar at 12 C., then 1 day at 35 C., Heidenhain stain; D, *B. diploides*, 2 weeks on agar at 12 C., then 1 day at 35 C., stained with fuchsin; E, the same, Heidenhain stain; F, *B. diploides*, 4 days on agar at 18 C., stained with fuchsin; G, *B. diploides*, 2 weeks on agar at 12 C., then 1 day at 35 C., stained with fuchsin.

TOXICO-IMMUNOLOGIC AND SEROLOGIC RELATIONSHIP OF *B. BOTULINUS*, TYPE C, AND *B. PARABOTULINUS*, "SEDDON." XXII.

W. PFENNINGER

From the George Williams Hooper Foundation for Medical Research, University of California Medical School, San Francisco

During the past few years, organisms forming soluble toxins which in susceptible animals produce symptoms and pathologic changes indistinguishable from those noted after the administration of botulinus toxin, types A or B, have been found in widely divergent localities. The organism isolated by Bengtson¹ and Graham² in the United States, and known as *B. botulinus* type C, was first isolated from the larvae of the green fly *Lucilia seratica*, later from the contents of the crops of chickens, which had died of "limberneck" and from the stomach content of a horse. The Australian type culturally and biochemically almost similar to the type C strains was isolated by Seddon³ from the bone marrow of a fatal case of so-called midland cattle disease in Tasmania. He called the organism "*B. parobotulinus*" and considered it of etiologic importance. Midland cattle disease appears to be identical with impaction paralysis found in Victoria, with "dry bible" in South Australia and also with lamziekte of South Africa.

Biochemical, cultural and toxicologic studies of type C strains have been reported by Bengtson. She believes that the type C is more closely related to the original van Ermengem culture than are the A or B strains. A preliminary study has shown that the C type strains cannot be distinguished morphologically or culturally from the *B. parobotulinus* strain. It will be shown in a later paper that the two species are so nearly alike in their metabolism that they cannot be differentiated by the ordinary chemical changes produced in culture mediums. The following questions therefore arose: Are the type C and the "Seddon" strains serologically and toxicologically alike? If not, is there any relationship between them and the A and B types?

Received for publication, June 2, 1924.

¹ Pub. Health Rep., 1922, 37, pp. 164 and 2252.

² Jour. Am. Vet. Med. Assn., 1924, 64, p. 723; abst., Bacteriol., 1923, 7, p. 29.

³ Jour. Compar. Path. & Therap., 1922, 35, p. 147.

The 6 type C strains used in this study were originally obtained from Miss Ida B. Bengtson.⁴ In a note accompanying the cultures, it was stated that one strain "Saunders" was probably nontoxic. According to tests carried out in this laboratory, this seems to be the case; another strain, 117, was also found to be nontoxic or only feebly toxic. The beef heart cultures of the other strains produced typical symptoms of botulism after subcutaneous inoculation in mice and in guinea-pigs.

The original "parabotulinus" strain and the corresponding antitoxin was received from Dr. H. R. Seddon in November, 1922.

The first question asked was: Is the toxin produced by the "Seddon" strain identical with the type C toxin? To answer this question, a few toxin-antitoxin tests were made on guinea-pigs. Animals were injected with toxin-antitoxin mixtures in the following manner: approximately 2 MLD of the filtered toxin of strain C 121 was mixed with 0.2 or 0.5 c c. of antitoxin, types A, B, C and "Seddon" antitoxin, respectively; after standing at room temperature for one half hour, the mixtures were inoculated subcutaneously into guinea-pigs.

TABLE 1
NEUTRALIZING EFFECT OF TYPE A, B, C OR SEDDON ANTITOXIN ON TYPE C TOXIN

FILTERED TOXIN C 121 (FROM AN 11-DAY OLD BEEF HEART CULTURE)						
Guinea-Pig	Weight in Gm.	Dose in C c. of Toxin in 1:25 Dil.	Antitoxin		Symptoms After	Death After
			Type	Dose in C c.		
4	400	0.90	A	0.5	20 hrs.	24½ hrs.
1	460	0.92	B	0.5	20 hrs.	28½ hrs.
2	250	0.56	C	0.2	No reaction	13 days intercurrent infection
3	450	0.90	Parabot. "Seddon"	0.5	44 hrs.	59½ hrs.
5	480	0.96	20 hrs.	24½ hrs.
PARABOTULINUS "SEDDON" (FROM AN 11-DAY OLD BEEF HEART CULTURE)						
6	300	0.6	A	0.5	43½ hrs.
7	550	1.1	B	0.5	45½ hrs.
8	340	0.68	C	0.2	No reaction	Survived
9	460	0.92	B.parabot. "Seddon"	0.5	No reaction	Survived
10	320	0.64	19½ hrs.

That C type toxin is neutralized only by C type antitoxin, is shown in the results listed in table 1. Guinea-pigs inoculated with either type A, B or "Seddon" antitoxin died in about the same time as the control.

⁴ Public Health Reports, 1923, 38, p. 340; strains 117, 121 and 3421 Saunders and 487 (limberneck) and 526 (stomach of a horse).

Filtered parobotulinus toxin (2 MLD) treated in the same manner and inoculated into guinea-pigs gave entirely different results.

Both type C and "Seddon" antitoxin protect guinea-pigs against the parobotulinus toxin, while type A and B antitoxin have no neutralizing effect. Antitoxin type C prepared by Bengtson or any Type C serum, according to the latest studies of Graham and Boughton,⁵ protects animals against type C and parobotulinus toxin. In this study it has, however, been demonstrated that the Seddon antitoxin fails to give cross protection for C strains, but neutralizes its homologous toxin. P. Schoenholz of this laboratory has previously observed the cross protection on mice afforded by type C antitoxin against parobotulinus toxin.

TABLE 2

TOXIN ANTITOXIN TESTS WITH TOXIN PRODUCED BY GROWTH OF *B. BOTULINUS* TYPE C WITH SEDDON AND TYPE C ANTITOXIN

Guinea-Pigs	0.1 C c. Toxin Produced by Strain; Mixed with 0.5 C c. Seddon Antitoxin and Injected Subcutaneously	Result	Guinea-Pigs	0.1 C c. Toxin Produced by Strain; Mixed with 0.2 C c. Type C Antitoxin and Injected Subcutaneously	Result
1	C 3421	Dead 42 hrs.	6	C 3421	Survived
2	C 117*	Survived	7	C 117	Survived
3	C 526	Dead 40 hrs.	8	C 526	Survived
4	C 487	Dead 40 hrs.	9	C 487	Survived
5	C "Saunders"***	Survived	10	C "Saunders"	Survived

* These strains according to the tests thus far carried out in this laboratory are nontoxic.

When unfiltered toxic cultures of the other C strains at our disposal were mixed with type C and with "Seddon" antitoxin, respectively, the same relationship as was shown by the filtered C type toxin (121) was found to exist, e. g., C antitoxin protected, "Seddon" antitoxin gave no protection. It will be interesting to determine whether other lots of C type antitoxin show the same cross protection for parobotulinus toxin as was shown by the sample tested in these experiments. The confirmation of these observations with additional parobotulinus strains and other preparations of type C antitoxins will be of great interest from a practical as well as from a theoretical point of view.

The antigens used for the immunization of rabbits and for the agglutination tests were prepared according to the method described by Schoenholz and Meyer.⁶ A method for the production of agglutinating serums similar to that described by these investigators was used in the

⁵ Jour. Am. Vet. Med. Assn., 1924, 64, p. 723.

⁶ Jour. Infect. Dis., 1923, 32, p. 417.

production of the antisera with the C strains. The rabbits received one small dose intravenously on the first day followed by two larger doses on the 7th and 14th day, respectively. Five days after the last injection, the animals were bled from the heart. Particularly potent antisera were produced in 3 rabbits, the titer ranging from 1:9,000-

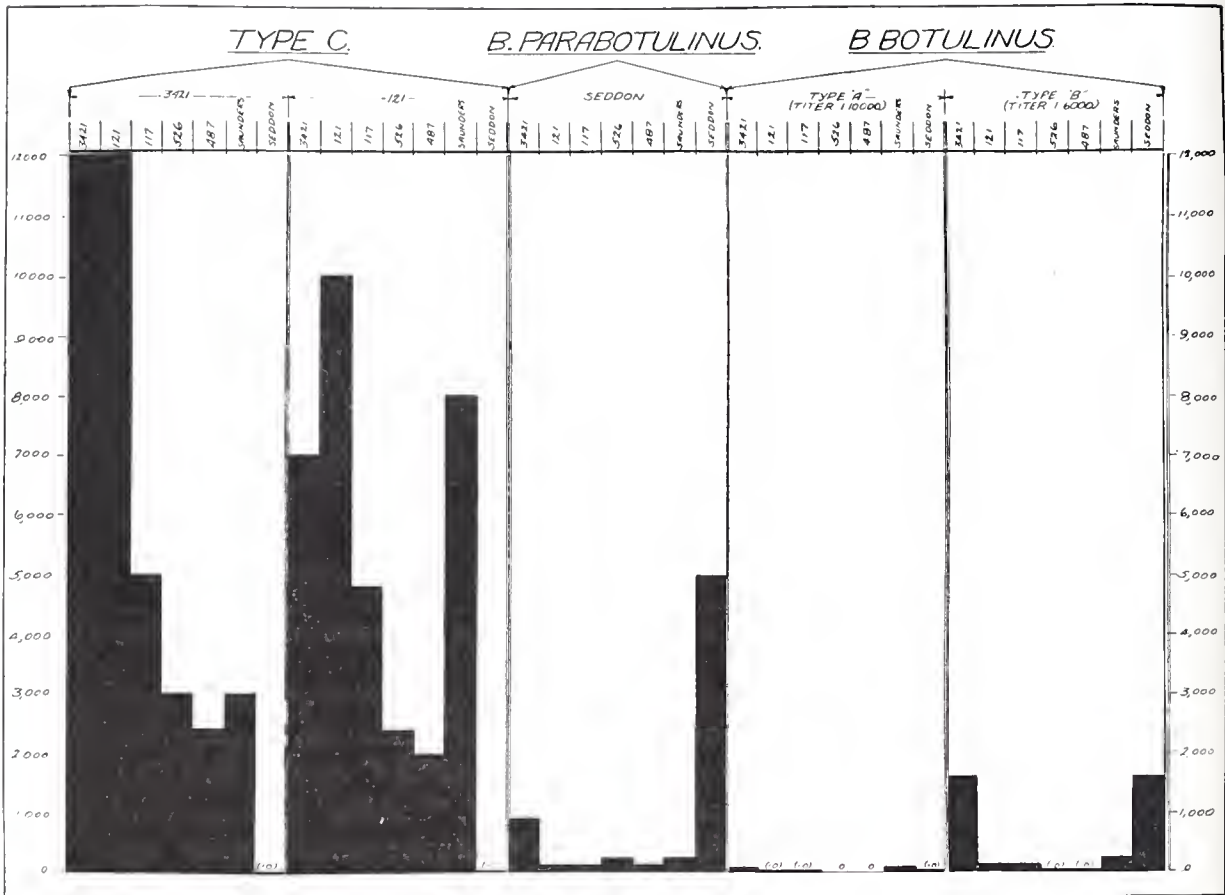


Chart 1.—Agglutination reactions of *B. botulinus* type C and parobotulinus "Seddon."

1:12,800, while the titer of the 4th, although satisfactory, was somewhat lower, e. g., about 1:4,000.

The "Seddon" antiserum was produced by a quick method of immunization, e. g., 3 inoculations were given intravenously on 3 successive days. This treatment was tolerated well and gave satisfactory results.

When antigens of the C types and the parobotulinus strain were titrated with the antisera prepared from strains C 3421 and C 121,

respectively, and with the antiserum prepared from *B. parabolulinus* "Seddon," an interesting relationship was noted.

Antiserums prepared from C 3421 or C 121 agglutinated all of the C type antigens. However, they did not all react to full titer. Results plotted in chart 1 show that antigens prepared from strains C 3421 and C 121 react to full, or almost full, titer with "C type" antiserums, that the 3 other strains—C 117, C 526 and C 487—agglutinated to from 20%-50% titer with both C type antiserums, while the 6th strain agglutinated to 25% titer with antiserum C 3421 and to 80% titer with antiserum C 121. It is interesting to note that one of these strains, "Saunders," is a nontoxic strain. Although the strain has lost its toxicologic properties, it has not lost its serologic identity. Similar observations which will be reported later have been made in this laboratory with several B type strains of *B. botulinus*. The six C type strains appear closely related, but whether they are serologically alike can be determined only by the absorption of agglutinins.

The parabolulinus antigen was not agglutinated by the 2 antiserums prepared with the C type strains, even in a dilution of 1:20. However, 5 of the C type strains were agglutinated by the "Seddon" antiserum in a dilution of 1:200, and one strain—C 3421—was flocculated even in a dilution of 1:800. The "Seddon" antiserum when absorbed with strain C 3421 was still capable of agglutinating the parabolulinus antigen, but the titer of the antiserum was somewhat reduced.

From these preliminary tests, it appears that the "Seddon" parabolulinus strain and the C type strains belong to different serologic groups, distinguishable from each other by means of the agglutination and the absorption test.

A few tests to determine whether a serologic relationship exists between the A, B, and C and so-called parabolulinus strains were made. An antiserum prepared from strain 38 A and one prepared from strain 67 B were tested with the 6 C type and the parabolulinus antigens. The A type antiserum reacted in very low dilutions with 4 of the 6 strains, while the B. parabolulinus strain was scarcely influenced. However, the B type antiserum gave marked coreactions, especially with strain C 3421 and *B. parabolulinus* Seddon. Both of these strains (chart 1) reacted in a dilution of 1:1,600. Further work along these lines will be carried out in the near future. Until then it will be impossible to discuss this interesting relationship.

CONCLUSIONS

B. paratuberculosis "Seddon" produces a botulinus-like toxin which can be neutralized by the botulinus type C antitoxin prepared by Bengtson; but the antitoxin prepared by Seddon for his paratuberculosis strain neutralizes only its homologous toxin.

Two type C agglutinating antisera failed to agglutinate the *B. paratuberculosis* even in low dilution. The antiserum prepared with the "Seddon" strain gives coreaction with type C strains in low dilutions.

BIOCHEMICAL ACTIVITIES OF *B. BOTULINUS*, TYPE C, AND *B. PARABOTULINUS*, "SEDDON." XXIII.

E. WAGNER

From the George Williams Hooper Foundation for Medical Research, University of California Medical School, San Francisco

In a previous communication,¹ the cultural findings of Bengtson² on one strain of *B. botulinus* type C (strain Saunders) have been confirmed by a series of quantitative chemical tests. The toxic anaerobe isolated from the larvae of *Lucilia caesar* produced little or no turbidity in broth, failed to disintegrate the meat particles in a beef heart mince, but evolved continuously a considerable amount of gas in various liquid mediums. Quantitative analyses left no doubt that the *Lucilia caesar* organism is chemically relatively inert. The immunologic studies conducted in this laboratory by W. Pfenninger³ offered an opportunity to make additional metabolic studies with 6 cultures of *B. botulinus* type C, kindly furnished by Miss T. C. Bengtson. A culture of *B. parobotulinus* "Seddon" was added to the series. This organism is related to the *B. botulinus* type C group, although it belongs to a distinct serologic subgroup.

The original program of experiments contemplated the application of quantitative plating methods in order to estimate accurately the number of viable organisms participating in the metabolic activities. Repeated attempts by Pfenninger to grow *B. botulinus* C strains on peptic digest blood-agar plates in anaerobic jars using the method described by Dozier⁴ were not successful. The cultures failed to grow on plates. In fact, the original cultures prepared in broth and transferred to liquid peptic digest mediums of high biologic value refuse to grow in dilutions as low as 1:10, and frequently they exhibited delayed development. A number of direct counts by means of a Helber chamber have been made, but the growth curves plotted with the figures thus obtained, although in the main consistent with the curves secured for *B. botulinus* types A and B, appeared rather irregular. Until supplementary studies have established some of the factors responsible for the

Received for publication, June 2, 1924.

¹ Jour. Infect. Dis., 1924, 34, p. 63.

² Pub. Health Rept., 1923, 38, p. 340.

³ Jour. Infect. Dis., 1924, to be published.

⁴ Ibid., 1924, to be published.

well-known variability in the reproduction of type C strains in liquid and solid mediums, it is deemed unnecessary to include the data on growth curves.

In addition to the chemical analyses, a few toxin tests were made. Previous tests had shown that *B. botulinus* type C may readily yield a toxin of 1:10,000 potency, although it is apparently nonproteolytic. This observation contradicted the statement of Heller⁵ that "the more proteolytic a pathogen is the more powerful is its toxin." On the other hand, it called attention to the opinion of the Medical Research Committee⁶ that "the truly proteolytic organisms are not those which produced potent toxin"; and the statement of Kendall, Day and Walker⁷ that, "so far as available information indicates true toxin formation appears to be incompatible with marked proteolysis." It is now established that the toxic *B. tetani* and *B. botulinus* are both proteolytic, and the conclusion of the last named writer is unquestionably too sweeping. However, chemical and toxicologic findings reported in this paper leave no doubt that *B. botulinus* type C may produce in certain mediums highly potent toxins, although successive nonprotein nitrogen determinations furnished figures which would classify the organism as feebly proteolytic or nonproteolytic. Furthermore, nontoxic cultures of the same bacteria have been analyzed, and a definite increase in the nonprotein nitrogen fraction has been recorded. From the available knowledge, it must therefore be concluded that neither the statement of Heller nor the opinion of the Medical Research Committee is absolutely correct. Toxin production of a pathogenic anaerobic is not necessarily associated with bacterial proteolysis. Whether it is the result of autolytic processes as assumed by Dozier,⁴ deserves further investigation and will be considered elsewhere.

Mediums, Methods of Cultivation and Analysis.—Previous experiments have shown that a double strength veal infusion-peptic digest broth is the best medium for the study of differences in the nitrogen metabolism of anaerobic bacteria. Unhydrolyzed nitrogenous material in the form of a 1% gelatine was added to the medium. It was assumed that the gelatine stimulated the enzymatic activity of the growing organisms. The findings made on this medium have been checked by analyses of cultures produced in the peptic digest-beef heart mince used as a standard medium for anaerobic work in this laboratory. In a series of tests, glucose has been added to the veal infusion-peptic digest-gelatine broth. Preliminary studies indicated that sampling of the cultures at frequent intervals was unnecessary. The contents of single flasks containing 150 c.c. of medium and not portions of a 2 liter culture as practiced in the experiment with *B.*

⁵ Ibid., 1920, 27, p. 383.

⁶ Brit. Med. Res. Com. Special Report, 1919, Ser. 39.

⁷ Jour. Infect. Dis., 1922, 30, p. 141.

botulinus type A, B. sporogenes and B. tetani,¹ have been analyzed. The broth was stratified with petrolatum and sterilized at 110 C. for 40 minutes. At least 3 flasks were seeded with 2 c.c. of an 18-24 hour old culture of the various strains grown in the identical medium which was used for the metabolism studies. Smaller inoculums frequently failed to grow. Repeated attempts to develop active cultures from heated spores were equally unsuccessful. The chemical analysis took into consideration the following metabolic products: amino acids, ammonia, and nonprotein nitrogen; also the reaction. Little need be said with regard to the technic employed in carrying out these analyses, since the same methods previously described have been used. However, since special emphasis will be placed on the nonprotein fraction, the exact procedure for its determination is herewith detailed. Fifty c.c. of the culture were placed in 250 c.c. volumetric flasks, diluted, and the complex nitrogen compounds precipitated with 25 c.c. of 10% sodium tungstate and $\frac{2}{3}$ N. H_2SO_4 according to the Folin technic. A readjustment of the reaction to congo red was often necessary on account of the high buffer value of the medium. If in any way possible, duplicate precipitations were made. Triplicate samples of 50 c.c. each of the filtrate were used for analysis, and the nitrogen determined by Kjeldahl as usual. Toxin tests have been made on mice; either by intraperitoneal injections or by feeding.

EXPERIMENTAL DATA

Before describing the actual chemical changes produced by the organisms, it appears desirable to record briefly the macroscopic appearance of the mass cultures. As already indicated, the growth of the type C strains was frequently spasmodic; for example, strain 526 (series 49) was the only one which grew regularly. One flask of the series of 3 seeded with strains 117, 3421, 487 and Saunders failed to reveal any definite growth. Transplants into meat mash mediums showed similar results. Ornstein,⁸ Bengtson and others have noted this peculiar behavior of certain B. botulinus cultures.

When growth took place in veal infusion-peptic digest-gelatin, it was regularly indicated by a marked turbidity. At the end of 48 hours, the culture consisted of a crystal clear, golden yellow supernatant fluid and a flocculent precipitate, which was readily distributed in the broth by agitation. The parabotulinus strain failed to render the broth turbid, but grew in a moundlike stratum inclosing about one half of the medium in a flask. This peculiar turbidity settled in a few days and formed a sediment similar to that of the true type C cultures. The types A and B cultures clarified more slowly, formed a dark sediment and rendered the medium slightly paler in color.

The gas production of the Seddon and type C strains was in no case equal to that of either type A or B cultures. The peculiar, offensive odor of the B. botulinus gases was absent, with the exception of the cultures of strain 3421 and "Seddon," in which the presence of a volatile sulphur compound was repeatedly noticed. In the meat mash, slight turbidity of the broth was accompanied by continuous gas production from around the meat particles. The latter remained unchanged in color and form.

In previous papers, the chemical activities of B. botulinus types A and B have been described. Attention is therefore mainly directed toward the behavior of the type C strains. The data on the types A and B cultures simultaneously collected will be considered only for purposes of comparison. The chemical analyses are present in Table I.

The type C strain produced in the veal infusion-peptic digest cultures a slight increase in the amino acid nitrogen fraction; on the 10th day approxi-

⁸ Ztschr. f. Chemotherap., 1912, 1, p. 458.

TABLE 1

THE NITROGEN CHANGES PRODUCED BY VARIOUS TYPES OF B. ROTULINUS

Culture	Time Days	Double Strength Veal Infusion — Peptic Digest + 1% Gelatine										Meat Mash—Peptic Digest				
		Without Glucose (Series 49)					With Glucose (Series 49)					Series 50				
		Amino Nitro- gen, Mg.	Am- monia Nitro- gen, Mg.	P _H "C"	Non- pro- tein Nitro- gen, Mg.	Increase in Non- protein Nitro- gen, Mg.	M. L. D. for Mice, C c.	Glucose, %	Glucose Used, Mg.	P _H "C"	Amino Nitro- gen, Mg.	Am- monia Nitro- gen, Mg.	P _H "C"	Non- pro- tein Nitro- gen, Mg.	Increase in Non- protein Nitro- gen, Mg.	M. L. D. for Mice, C c.
Control	0	66.4	20.5	7.6	195.8	3.33	0	6.6	61.1	14.6	7.4	160.5
	4	61.4	18.8	7.6	195.8	0.0	3.33	0	6.6	63.3	14.0	7.4	173.2	12.7
A Strain 97	10	66.0	20.1	7.6	219.9	14.1	—	—	—	—	—	—	—	—
	4	108.6	186.7	6.7	406.9	258.1	0.735	2,595	5.8	78.1	162.7	6.6	420.3	259.8
B Strain 43	10	87.0	213.9	6.9	406.9	247.0	—	—	—	—	—	—	—	—
	4	127.3	184.5	6.7	476.0	280.2	1.37	1,360	6.8	—	—	—	—	—
C Strain 117	10	101.2	210.1	6.7	445.0	225.1	—	—	—	—	—	—	—	—
	4	77.0	20.9	6.7	215.4	19.6	2.94	330	5.0	—	—	—	—	—
C Strain 526	10	85.4	21.7	6.5	228.9	9.0	—	—	—	—	—	—	—	—
	4	70.4	18.5	6.6	201.8	6.0	2.94	330	5.0	68.8	16.6	6.5	179.3	18.8
C Strain 3421	10	82.1	21.7	6.5	232.9	13.0	0.005—	—	—	—	93.4	18.5	6.5	180.8	7.3	0.5—
	30	—	—	—	—	—	—	—	—	19.5	6.4	229.5	56.3	0.5—
C Strain 121	4	136.9	40.3	6.7	292.2	96.4	2.84	490	5.3	67.9	15.3	6.5	210.0	49.5
	10	—	—	—	—	—	0.005—	—	—	—	90.9	17.6	6.4	227.5	51.3	0.5—
C Strain 487	4	69.1	19.8	6.6	195.8	0.0	3.33*	0	6.6	42.5	14.1	6.5	170.2	9.7
	10	72.0	23.8	6.5	262.1	42.2	0.005+	—	—	—	79.3	21.7	6.4	176.2	3.0
C Strain 487	4	78.8	20.7	6.6	—	—	2.97	300	5.0	67.8	16.6	6.5	185.3	24.8
	10	99.1	33.8	6.5	262.0	42.1	0.005+	—	—	—	80.1	20.1	6.4	179.3	6.1	0.5—
Para- botulinus Seddon	30	—	—	—	—	—	—	—	—	20.1	6.4	222.0	48.8	0.5+
	4	70.2	19.6	7.2	180.5	0.0	2.90	430	5.0	93.5	29.4	6.5	265.1	94.6
C Strain Saunders	4	—	—	—	—	—	—	—	—	106.5	30.9	6.4	247.1	73.9	0.05++
	30	—	—	—	—	—	—	—	—	33.2	...	301.3	128.1	0.05++
C Strain Saunders	4	70.2	19.6	6.6	219.9	24.1	2.87	460	5.0	—	—	—	—	—
	10	95.5	23.8	6.5	259.0	39.1	—	—	—	—	—	—	—	—

* No growth.

mately 22 mg. were formed. Strain 3421 behaved in series 49 like a type A or B strain, although the transplants indicated a pure growth. In subsequent cultures, this strain was less active and acted like a true type C or parobotulinus strain. The analysis of the 4-day old culture of strain 121 showed a loss in amino acid; similar depletions of these acids have previously been noted in young cultures of *B. botulinus*, types A and B.

The ammonia production is very low in the type C cultures, an average increase of 6.1 mg. corresponding to a conversion of 1.2% of the total nitrogen into ammonia. The types A and B cultures indicate a 40% conversion.

The mediums contained 476 mg. (series 49) and 435 mg. (series 50), respectively, of nitrogen per 100 c.c., more than half of which was precipitated by tungstic acid. Types A and B strains converted inside of 4 days the complex nitrogen molecules of the medium to soluble nonprotein nitrogen compounds. The type C and the parobotulinus strain induced slight changes in the non-protein fraction. The increase was almost negligible; approximately 42 mg. was the maximum recorded. These results leave no doubt that *B. botulinus* type C and parobotulinus are feebly proteolytic in veal infusion-peptic digest-gelatine. According to Dernby,⁹ all bacteria probably contain proteolytic enzymes, although their effect cannot always be demonstrated. Kendall and his associates¹⁰ found that organisms like *B. coli*, *B. typhosus* and the Shiga bacillus grow at the expense of the "polypeptid" nitrogen of the medium, which on prolonged incubation is restored to the medium from the bacillary bodies by autolytic processes.

The endocellular activity of *B. botulinus* has been demonstrated by a number of experiments, for example:

Four liters of an 18-hour old broth culture of *B. parobotulinus* "Seddon" furnished on centrifugalization a bacterial sediment (204 mg. of nitrogen) which was resuspended in salt solution, mixed with toluene and incubated at 37 C. The bacteria showed progressive disintegration; the percentage of gram-negative, faintly staining, rods increased from 5 to 98% in 4 days. The total nitrogen of the centrifugalized salt solution increased from 18 to 25.6 mg. per 100 c.c., and the amino nitrogen from 4.5 to 8 mg. per 100 c.c. These changes were absent in a specimen of the same suspension heated at 70 C. for one hour. As it was impossible to increase the bacterial yield and the total amount in nitrogen of the bacterial bodies, the methods of Sturges and Rettger¹¹ have not been applied. As a whole, the chemical changes in the autolyzing suspensions are slight, but they are sufficiently definite to indicate that enzymatic disintegration takes place. It is recalled that in the C and parobotulinus cultures the nonprotein nitrogen is formed largely between the 4th and 10th days, while previous work with *B. botulinus* types A and B, *B. sporogenes* and *B. tetani* has shown¹²

⁹ Biochem. Ztschr., 1922, 126, p. 105.

¹⁰ Jour. Infect. Dis., 1922, 30, p. 239.

¹¹ Jour. Bacteriol., 1922, 7, p. 551.

¹² To be published.

that the increases of soluble nitrogen accompanies the active proliferation of the organisms. The A and B cultures contain active gelatine liquifying exo-enzymes which are not demonstrable for the C and "para" types. However, it is not unlikely that the delayed nitrogen changes in the C and "para" cultures are due both to the disintegration of the bacterial bodies and the subsequent action of the endo-enzymes set free by this process. These observations lend some support to the contention of Diehl¹³ that enzymes are not preformed in the bacterial cell but are dependent for activation on the constituents of the medium.

An addition of glucose increased slightly the gas production of the C strains. However, the sugar consumption was only one-fifth to one-sixth of that determined for *B. botulinus* type A and B. This behavior is indicative of a lowered metabolic rate and is characteristic for all the type C and parabolulinus strains investigated.

In the meat mash cultures (series 51), slight changes in the amino acid and ammonia were noted, but a marked increase in the nonprotein fraction was most striking. Approximately one-third of the amount produced by a type A strain was formed in 3 type C. cultures. Strain 121 failed to grow, and therefore served as an excellent control. It is rather suggestive that certain substances of the meat mash activated the autolytic enzymes in the meat more readily than in the veal infusion gelatine. This interpretation of the increased nonprotein nitrogen is in accordance with the views previously detailed.

The reaction of the medium changed from P_H 7.0-7.2 to P_H 6.4; the volatile or fixed acids were not neutralized by alkaline split products. Filtrates of the type C cultures failed to coagulate milk or to liquefy gelatine; the strains produced neither a rennet ferment nor a gelatinase.

Systematic toxin tests were not conducted on account of the fact that some of the type C strains failed to produce toxins on the mediums used for the chemical analyses. With the exception of *B. parabolulinus*, every culture either lost its toxicity or formed the poison only on prolonged incubation. These observations were at first disturbing, although it was known from the studies of Bengtson that type C cultures may contain two bio types, a toxin and a nontoxin-producing type. With the aid of agglutination tests, the nature and the purity of the nontoxic cultures 526, 3421 and 487 were established and controlled. The tests conducted on the cultures used in series 49 and 50 suggested some relationship between the potency of the toxin and the yield of non-

¹³ Jour. Infect. Dis., 1919, 24, p. 347.

protein nitrogen in the centrifugalized broth. The toxin tests of the meat mash cultures, however, proved the reverse—that the toxicity of the cultures was not dependent on the nonprotein nitrogen. For example, the highly toxic “Seddon strain” showed an increase of non-protein nitrogen of 131.1 mg., while the nontoxic type C strain 3421 (serologically confirmed and pure) formed 146.9 mg. of soluble nitrogen. The results are quite conclusive. An increase of the non-protein nitrogen fraction of more than 100 mg. per 100 c.c. of medium in a *B. botulinus* type C culture does not necessarily prove its ability to generate a typical toxin. In fact, it is shown that toxin may be present in a culture which shows an insignificant increase in soluble nitrogenous substances. Toxin formation is therefore not necessarily associated with definite proteolysis.

The biochemical studies conducted with *B. botulinus* type C have unexpectedly confirmed the early studies of van Ermengem, Forssman and others. A perusal of their publications leaves no doubt that the strains of *B. botulinus* investigated by them were either type C strains or closely related species. Variability and inconstancy in toxin production dependent on composition and reaction of the mediums are frequently emphasized. To the bacteriologist who has studied types A and B strains, these reports appear unreliable. Toxin production by types A and B in any medium in which they grow is such a constant phenomenon that any loss of this property is invariably attributed to a contamination of the stock cultures. Observations made in this laboratory on several type B cultures indicate that these orthodox views need revision. Prolonged artificial cultivation on unsuitable mediums exposed to the air may lead to nontoxic type B strains. It will be interesting to study these strains biochemically and to determine the factors which may transform toxic type B or C cultures to nontoxic strains.

CONCLUSIONS

The inability of type C and “para” strains to grow on anaerobic blood plates renders the estimation of the growth rate a difficult and complicated procedure. In liquid mediums the development is frequently spasmodic and thus far uncontrollable.

The changes in amino acid, ammonia and nonprotein nitrogen of C and parobotulinus strains are slight.

The ability of the organisms to autolyze has been demonstrated by microscopic and biochemical tests.

Exo-enzymes of the same character as found in types A and B are not demonstrable in the C cultures. The weak proteolytic activities are due to autolytic enzymes activated by the constituents of the medium.

The comparatively slight utilization of sugar is evidence of the low metabolism of types C and parobotulinus.

The toxin production of a pathogenic sporulating anaerobe is not necessarily associated with bacterial proteolysis.

EFFECT OF DIRECT SUNLIGHT, DIFFUSE DAYLIGHT AND HEAT ON POTENCY OF BOTULINUS TOXIN IN CULTURE MEDIUMS AND VEGETABLE PRODUCTS. XXIV

P. SCHOENHÖLZ AND K. F. MEYER

*From the George Williams Hooper Foundation for Medical Research, University of California
Medical School, San Francisco, California*

Van Ermengen,¹ Thom, Edmondson and Giltner,² Burke, Elder and Pischel,³ Orr⁴ and others have reported observations on the destructive action of direct sun and diffuse daylight and of heat on the toxin elaborated by *B. botulinus* in various mediums. In the light of recent epidemiologic studies, it appears advisable to broaden the findings of these workers by additional experiments. The data may be conveniently presented under three different headings:

(A) The destruction of the toxin by exposure to sun and to diffuse daylight.

(B) The destruction of the toxin produced by the growth of the anaerobe in culture mediums in preserved vegetables by exposure to a temperature of 80 C.

(C) The destruction of the disease-producing properties of toxic vegetables by heat applied to the canned products under conditions similar to those practiced in the household.

A. THE DESTRUCTION OF *B. BOTULINUS* TOXIN BY EXPOSURE TO SUN AND TO DIFFUSE DAYLIGHT

Experimental Series 1.—Sets of tubes containing 10 c.c. of toxic, unfiltered, but centrifugalized broth cultures⁵ of strains 80 (type A; M L D about 1:10,000) and 65 (type B; M L D about 1:100,000) and the liquors from two samples of home preserved asparagus were exposed to the action of direct sunlight and tested for the presence or absence of toxin at frequent intervals by the intraperitoneal injection of white mice. One tube of each set was covered with sterile petrolatum. The exposures were made during several days for from 3½ to 8 hours at a time; in the meantime, they were kept in the dark in the ice chest. The first 90 hours of exposure were made during the months of May and June.

After 27 and 56 hours' exposure, 0.5 c.c. of the vegetable liquor and broth toxins produced typical symptoms in from 2½ to 4 hours. After 90 hours' exposure, one sample of asparagus was no longer toxic. After 118 hours, the poison in the second sample of asparagus and that produced by strain 65 (type B) had been destroyed, but the specimens of the liquors, which had been covered at the beginning of the experiment with sterile petrolatum, were still toxic. Toxin No. 80A either when protected from or exposed to the action of air remained

Received for publication, June 2, 1924.

¹ Ztschr. f. Hyg. u. Infektionskrankh., 1897, 26, p. 1.

² Jour. Am. Med. Assn., 1919, 73, p. 907.

³ Arch. Int. Med., 1921, 27, p. 265.

⁴ Jour. Med. Res., 1920-1921, 42, p. 127.

⁵ The method of preparing the mediums will be found in succeeding paragraphs.

virulent for animals. Lack of sufficient test material prevented further inoculations of animals. During the experiment, the temperature fluctuations caused by the sun rays in the vicinity of the tubes were recorded by a Tycos thermograph; they varied from 22.2 C to 42 C., averaging 31.5 C. The highest temperatures were recorded for about 6 hours during the first 3 days of exposure and not at the end of the period. Exposure to such a temperature (40-42 C.), for so short a period, is probably a subordinate factor responsible for the deterioration of the toxin. Fermi and Pernossi⁶ have shown that an increase in temperature from 37 C. to 56 C. hastens the destructive action of direct sunlight on tetanus toxin. Comparative tests to determine whether a temperature above 40 C. for a short period of time hastens the destructive action of light on the botulinus toxin were not carried out. From the experiments to be reported in the subsequent paragraphs it is indicated that probably oxidative processes, in combination with the sun rays, irrespective of the temperature at which the liquors are exposed, inactivate the toxic fluids.

Roux and Yersin,⁷ working with filtered diphtheria toxin, made some observations which have a bearing on this subject. They found that the destructive action of air is very slow in the absence of light, but it is much more rapid in the presence of direct sunlight. Filtered diphtheria toxin, when exposed to air and to direct sunlight for 5 hours, produced only a local edema at the site of inoculation. On the other hand the potency decreased only slightly when the toxin was exposed to sunlight for 10 hours but was protected from the air. Fermi and Pernossi⁶ found that dried tetanus poison dissolved in water is destroyed in from 8 to 10 hours by the action of direct sunlight. The highest temperature reached was 56 C. When the tubes were kept in water and held at 37 C., the toxin was destroyed in 15 hours. Thom, Edmondson and Giltner,⁸ working with a botulinus toxin containing 200 M L D per c.c., report that a 24 hours' exposure to the action of direct sunlight failed to destroy the toxic glucose beef-infusion broth filtrate of the Boise strain, but a 40 hours' exposure rendered it completely inert.

Further investigations are necessary to elucidate the factors responsible for the difference in photoresistance of botulinus toxin reported in the literature. At least, it would be interesting to expose to sun or diffuse light toxic food products hermetically sealed in jars and determine systematically the disappearance of the poisonous properties. The observations of Roux and Yersin, Fermi, and others, invite comparative studies with dried and soluble toxins. Some unpublished observations made in this laboratory have shown that toxic alfalfa hay, dried and exposed to sunlight, fog and other weather condition remained toxic for 19 days. The findings reported in this paragraph must be considered as preliminary, and deserve further elaboration.

Experimental Series 2.—In order to test the destructive action of diffuse daylight, three samples of whole toxic asparagus liquors were kept covered with a layer of sterile paraffin oil⁸ both in test tubes and in brown bottles and placed

⁶ Centralbl. f. Bakteriöl., I, 1894, 15, p. 303.

⁷ Ann. de l'Inst. Pasteur, 1889, 3, p. 273.

⁸ It is generally believed that the toxin of *B. botulinus* retains its strength just as that of *B. tetani* when stratified with paraffin oil. Experimental studies have thus far not been published. In this series, the paraffin oil prevented the evaporation of the toxin fluids.

at room temperature at a window with northern exposure and reflected light during the months of November-July. Duplicate samples were held as controls in the dark at icebox temperature (average 8 C.). The test for the potency of the toxin were made on guinea-pigs (chart 1).

It will be noted from the data in chart 1 that during 4½ months the potency of one sample held at icebox temperature fell to about 0.1 of its original strength; in a 2d, it remained constant, while the 3rd showed a slight deterioration. Three and one-half months later, or after 8 months, the first had become slightly weaker (a dilution of 1:1,000, killed a guinea-pig in 8 days), the 2d retained its original

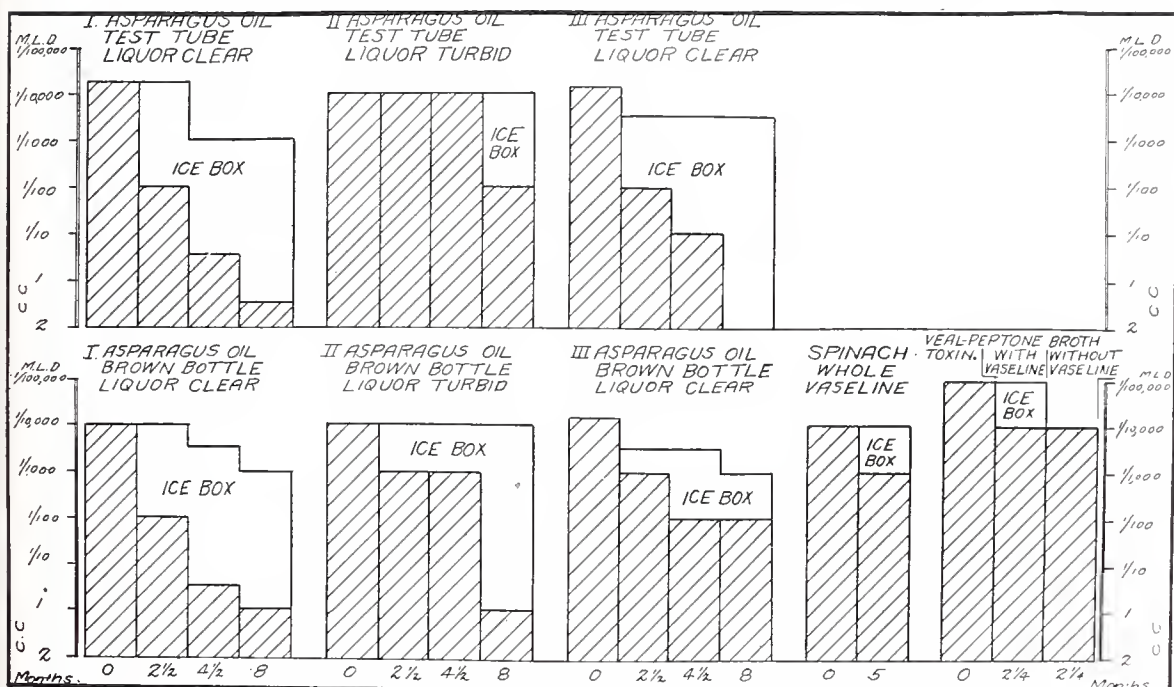


Chart 1.—Effect of diffused daylight and room temperature [20 C.] on *B. botulinus* toxin in vegetable liquors and veal peptone broth.

strength, while the 3rd showed no further decline in potency. At room temperature and exposed to daylight, the deterioration was more rapid. Even after 2½ months' exposure, the potency of all but one liquor dropped from one-tenth (in 2 brown bottles) to one-hundredth of the original strength. After 8 months' exposure, only a trace of toxin could be demonstrated in the first sample, while 1 c.c. of a dilution of 1:100 of the second was fatal to a guinea-pig in 6 days after subcutaneous inoculation; the 3rd was nontoxic. The latter toxin kept in a brown bottle instead of a test tube at room temperature and diffuse daylight was still toxic in a dilution of 1:100.

Whole toxic spinach and centrifugalized glucose broth toxins prepared from strains 80 (type A) and 65 (type B) were similarly exposed in test tubes and examined at varying intervals. In this experimental series, one of each set was covered with sterile petrolatum. During 5 months' exposure to diffused light at room temperature, the potency of every sample of spinach had decreased slightly. The same specimen held at icebox temperature for the same period of time retained its original strength. The broth toxins deteriorated to about one-tenth of their original strength during 2¼ months' exposure to diffuse light and air. When protected from the action of air, no significant loss in toxicity could be detected during the same period of time. Their strength remained the same in the icebox at a low temperature and in the dark.

Gates and Olitsky,⁹ Hall¹⁰ and others have shown that liquid paraffin oil has little value as an effective seal against the absorption of oxygen by a liquid medium. The rate of destruction of the toxin in liquors exposed to the action of air, when compared to the rate of deterioration of those kept under a petrolatum seal indicates that the destructive process is at least in part an oxidative one. In this connection, it should be pointed out that the color of the broth toxins exposed to air and sun or daylight become dark in a short period of time, while those protected from the action of air but exposed to sun or daylight retain their original color.

Summary.—The toxin produced by the growth of *B. botulinus* in glucose peptone veal infusion broth and in vegetables may be destroyed by exposure to the action of direct sunlight and air in about 90-118 hours. When the poison is kept anaerobically by a layer of sterile petrolatum, it remains toxic for a longer period of time.

Exposure of broth poison or of toxic vegetable products to the action of diffuse daylight and air at room temperature causes a slow but progressive loss of potency. A marked reduction in toxicity usually takes place in about 2½ months. When protected from the action of air or from the action of light, the rate of destruction of the poison is decreased. When vegetable toxins were kept in the dark in the ice box, only a slight loss in potency could be noted over a period of from 5 to 8 months.

⁹ Jour. Exper. Med., 1921, 33, p. 51.

¹⁰ Jour. Bacteriol., 1921, 6, p. 1.

B. THE DESTRUCTION OF THE TOXIN PRODUCED BY THE GROWTH
OF *B. BOTULINUS* IN CULTURE MEDIUMS AND IN CANNED
VEGETABLES BY EXPOSURE TO A TEMPERATURE OF 80 C.

According to van Ermengem,¹ the watery extract of the toxic ham responsible for the Elzevelles outbreak was weakened by an exposure to a temperature of 58 C. for 3 hours, while the temperature maintained at 80 C. for 30 minutes rendered the toxin practically inactive for guinea-pigs. However, 10 c.c. of such a heated filtrate when fed to a rabbit produced cachexia and diarrhea, and after several weeks the animal succumbed. It is not unlikely that the toxin which resisted a temperature of 80 C. is one of the heat resistant substances as found by Smith and Ten Broeck¹¹ and others in the representatives of the colon-paratyphoid group. Roux and Yersin⁷ found a heat resistant residue in filtered diphtheria toxin. Rabbits, which received intravenously or subcutaneously large quantities (35 c.c.) of toxin heated at 100 C. for 20 minutes showed no immediate effect, but usually succumbed after varying intervals of time. Several days before their death they showed paralysis, principally of the posterior extremities. They believe that this residue is similar to the sterile filtrates of the organs of fatal cases of diphtheria. According to Landmann,¹² exposure to a temperature of 75 C. for one hour destroyed the poison, while Leuchs,¹³ working with the toxin produced by both the Elzevelles and Darmstadt strains, stated that complete destruction takes place by heating the toxin at a temperature of 80 C. for 30 minutes. Armstrong, Story and Scott,¹⁴ working with toxic olives, reported similar observations. Shippen,¹⁵ on the other hand, was able to inactivate the toxin produced by the growth of the Nevin strain (type B) in symbiosis with yeast by subjecting it to a temperature of 65-70 C. for 20 minutes. Thom, Edmondson and Giltner² tested the filtered glucose beef infusion cultures of the Boise strain and reported that 30 minutes' heating at 62-66.5 C. exerted no appreciable effect on the toxicity of the cultures. However, a temperature of 68 C. applied for 30 minutes attenuated the poison, 10 minutes' heating at 70-73 C. rendered it inert, and at 75 C. it was destroyed in a few minutes. In a subsequent communication, Thom¹⁶ states that direct contact with a temperature of 85 C. will destroy the toxin in a short period of time. Randall¹⁷ reports that the lethal properties of botulinus toxin were not impaired by a temperature of 212 F. maintained for 10 minutes. A short series of tests carried out in a boiling water bath failed to corroborate these findings. Orr⁴ investigated the resistance of filtered toxins of relatively low potency produced by 10 different strains of *B. botulinus* in dextrose bouillon at various temperatures. The heated toxins were inoculated intraperitoneally into mice. He demonstrated that the resistance to heat varied greatly among the different toxins, but was independent of the potency or the type of the culture. At 72 C., the poison produced by every strain was destroyed in 2 to 18 minutes. The rate of destruction was from 4 to 8 times as rapid at this temperature as at 65 C. (10-85 minutes). At 80 C., the toxins were rendered inert in from 30 seconds to 5 minutes. The toxin produced by strain 11 (Nevin strain, type B) was apparently more resistant than any of the other toxins. Burke, Elder and Pischel³ experimented with the toxin developed

¹¹ Jour. Med. Res., 1914-1915, 31, p. 503.

¹² Hyg. Rundschau, 1904, 14, p. 449.

¹³ Ztschr. f. Hyg. u. Infektionskr., 1910, 65, p. 55.

¹⁴ Pub. Health Rep., 1919, 34, p. 2877.

¹⁵ Arch. Int. Med., 1919, 23, p. 346.

¹⁶ Am. Jour. Pub. Health, 1922, 12, p. 49.

¹⁷ Med. Rec., 1920, 98, p. 763.

in a can of artificially inoculated string beans. The liquor stratified with oil and held in a water bath at 80 C. for one hour remained toxic. In one experiment, the exposure of the bean juice for 5 minutes to a temperature changing from 70 C. to 100 C. and for 4 minutes at the boiling point did not completely detoxify it. In a third experiment, 1 c.c. of the same juice stratified with oil and exposed to 100 C. for 10 minutes was fatal to a guinea-pig in 96 hours. They did not determine whether death was due to the survival of a small amount of toxin or to the intoxication which followed the introduction of living spores. They emphasize the fact that vegetable juices, as for example uncentrifugalized and unfiltered bean juice, may require more heat than is recorded for the destruction of the filtered toxin produced in broth cultures.

The personal experiments dealing with this phase of the work may be divided conveniently into two groups: (*a*) those dealing with the destruction of the toxin prepared in glucose peptone veal broth or beef heart medium, (*b*) those dealing with the destruction of the toxin formed by the growth of the organism in canned or preserved vegetables.

Technic.—The mediums used for the production of the toxin were prepared as follows:

Five gm. of NaCl, 5 gm. dibasic potassium phosphate and 10 gm. of "Berna" peptone were added to 1 liter of double strength veal infusion. The reaction was adjusted to P_H 8 with 2/N sodium carbonate. A measured amount of the unfiltered medium was placed in an Ehrlenmeyer flask and autoclaved for 30 minutes at 15 pounds' pressure. Two per cent. glucose, in the form of a sterile 50% glucose solution and a layer of sterile petrolatum were added, and the mixture heated for another 20 minutes in the Arnold sterilizer. The flasks were incubated for 48 hours at 35 C. to test the sterility of the medium. The final reaction varied from P_H 7.6 to 7.8.

The beef heart medium was prepared by the method described by Dubovsky and Meyer,¹⁸ the reaction was adjusted to P_H 7.6. After inoculation, the cultures were grown for 10 days at 35 C., and then placed in the ice chest. After standing several days, the clear supernatant fluid could be pipetted off without disturbing the sediment. In order to guard against the presence of viable bacteria or spores in the supernatant fluid, the liquors were centrifugalized at high speed for 30 minutes. Filtration was avoided in order to prevent a loss in potency by adsorption in the candles. The strength of the toxin was determined by inoculating graded doses subcutaneously into guinea-pigs. That amount which proved fatal to a guinea-pig of approximately 250 gm. weight in about 96 hours, was regarded as the minimum lethal dose. Toxins prepared in the manner just described are very potent; 1 c.c. may contain 10,000 to 100,000 or more guinea-pig minimum lethal doses.

The toxic vegetable liquors were obtained either from naturally contaminated and spoiled products, or they were produced artificially by inoculating commercially canned foods with heated spores of *B. botulinus*, and grown at 35 C. for varying periods of time. Some of the liquors tested were secured from a series of experiments dealing with the production of toxin and signs of spoilage in canned goods artificially inoculated with detoxified spores of *B. botulinus*.¹⁹ The potency of a few of the liquors was low (M L D 1:10-1:100), but the remainder was just as toxic as the broth cultures.

A measured amount of the toxic broth culture or vegetable juice was pipetted into Pyrex tubes measuring about 7 mm. in diameter, with a 1 mm. wall, sealed

¹⁸ Jour. Infect. Dis., 1922, 31, p. 501.

¹⁹ Jour. Infect. Dis., 1923, 33, p. 289.

and held at icebox temperature. On the following morning, they were heated in a DeKhotinsky oil bath held at 80 C. The initial temperature was usually raised to from 81 C. to 83 C., and occasionally to 85 C., to allow for the usual fall after the immersion of the tubes. Within from 2 to 3 minutes the temperature fell to 79.5 C. The time was counted from the moment the tubes were immersed. Tubes were removed always after 2, 4, 6, 8, 10, 12, 15, and 20 minutes, and when testing vegetable toxins up to from 40 to 60 minutes at 5 to 10 minute intervals. After heating, they were cooled in water, then opened, and the toxicity tested by inoculating 1.7 to 2 c.c. subcutaneously into guinea-pigs, or 0.5 c.c. intraperitoneally into mice.

Several experimental series indicated that more reliable results may be obtained when the inoculations are made on guinea-pigs, since it appears that mice are decidedly sensitive to the inoculation of foreign matter. Apparently healthy animals, when inoculated intraperitoneally or even fed with certain types of suspected foodstuffs or heated cultures, may become sluggish and after an interval of from 2 to 5 days succumb with lesions of an acute gastro-enteritis. Furthermore, one series of tests in which 50 mice had been inoculated, had to be discarded on account of the high incidence of a preexisting paratyphoid

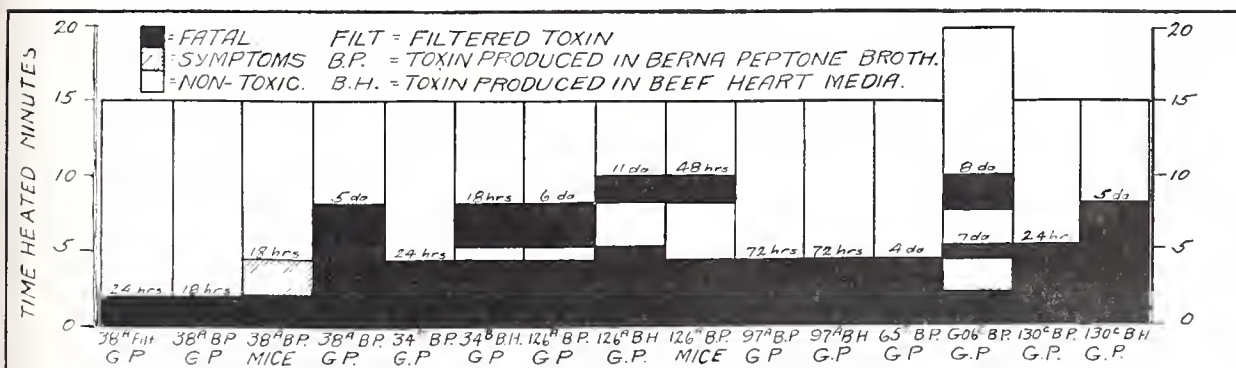


Chart 2.—Resistance of toxins produced in peptone veal broth and beef heart mediums heated at a temperature of 80 C.

infection in the stock animals. Although the symptoms produced in mice by the inoculation of *B. botulinus* toxin are quite characteristic,²⁰ they may, if slight, be masked by atypical gastro-intestinal disturbances. Since salivation is rarely noted and gross changes found at necropsy are not always pronounced, the interpretation of the results becomes difficult when weak toxins are tested. Guinea-pigs, however, are less sensitive to the injection of foreign matter, show typical symptoms and fairly characteristic lesions at necropsy.²¹ After several months' experience, it was concluded that the larger animals were more suitable for the tests required by the experiments to be reported. Consequently, later tests have been made entirely on guinea-pigs.

The results obtained in 15 sets of heat resistance tests with the toxins produced by 6 strains of *B. botulinus* and one strain of *B. botulinus* type C in glucose peptone broth and in beef heart medium are shown in chart 2.

The average resistance of the centrifugalized toxin to a temperature of 80 C. is 6 minutes. The greater part of the poison is destroyed in

²⁰ Bengtson: Pub. Health Rep., 1921, 36, p. 1665.

²¹ Necropsy findings described in ref. 18 on page 523.

the first few minutes; as a rule, after 4 minutes' exposure, only a trace of toxin is demonstrable, e. g., the inoculation of the heated juice is fatal to a mouse in 2 to 5 days or to a guinea-pig in more than 6 days. However, the toxin produced by two strains, viz., 126 (type A) and G 6 (type B) held at a temperature of 80 C. for 10 minutes remains toxic. In this connection, it is interesting to note that the most resistant toxin tested by Orr was prepared from a type B. (Nevin) strain. Likewise, one of the most resistant toxins tested in the series just reported was a B. type. The poison produced by the growth of the type C strain (130) in broth or beef heart medium survives from 6 to 8 minutes heating at 80 C.

A glance at the data plotted in chart 2 shows that a number of animals inoculated with heated toxins failed to show symptoms, although the samples, which were heated for longer periods, produced fatal intoxications. These irregular results or skips in the blocks recording the results were noted throughout the experiments. They were more frequent and more striking in the tests dealing with the vegetable juices. Various factors may be responsible for these inconsistencies. It is reasonable to suspect the varying susceptibility of mice as well as guinea-pigs to small doses of *B. botulinus* toxin. Observations made in this laboratory support this contention.²²

Irrespective of the irregular results recorded in these tests, it is evident that the broth toxins are rapidly destroyed at a temperature of 80 C. These findings confirm the published data of previous workers.

The experiments reported by van Ermengem on the filtered toxic, watery extract of ham, those of Burke, Elder and Pischel on contaminated toxic bean juice and some epidemiologic data analyzed in this laboratory, indicate that the toxic substances formed in the course of the growth of *B. botulinus* in animal and vegetable products is apparently more resistant to heat than that formed in peptone culture

²² In this connection, the following experiment carried out in a series of toxin-antitoxin neutralization tests is of interest. A dilution of standard botulinus toxin, kindly furnished by Dr. Ida Bengtson of the Hygienic Laboratory, U. S. Public Health Service, Washington, D. C., was made so that theoretically 1 c.c. contained 1 M L D of toxin. Seven guinea-pigs, whose weight varied from 200-340 gm. each, received exactly 1 M L D subcutaneously—the dose was kept constant irrespective of the weight of the animal. An eighth guinea-pig received 1 M L D calculated according to body weight. Only 1 of the 6 smaller guinea-pigs (200-270 gm.) died within the prescribed time; 2 showed slight symptoms and recovered, while the other 3 remained unaffected. When the dose for the heavier animal was varied in proportion to its weight, a fatal intoxication developed. Calculated on a basis of body weight, 3 guinea-pigs of less than 250 gm. weight received more than 1 minimum lethal dose of toxin. It appears, therefore, that either the actual increase in the amount of toxin injected was important in producing the fatal intoxication in the larger animal or that this particular one, in contrast to the smaller, was more susceptible to the intoxication. It may be concluded that the resistance of guinea-pigs as ordinarily purchased in the open market to small doses of the poison may vary considerably. These observations may serve as one of the possible explanations of the skip stops encountered in the toxin destruction tests; they also indicate that irregular results are to be expected when small laboratory animals are used to determine the presence of traces of botulinus toxin.

ducing properties of two samples of centrifugalized corn juice, although impaired, could still be demonstrated in appreciable amounts after an exposure of from 40 to 60 minutes at 80 C. A guinea-pig inoculated with the first specimen heated for 40 minutes died with typical symptoms of botulism in 66 hours. Toxicity in the other tests had been determined on mice; these animals, inoculated with samples heated for 30 to 60 minutes, died in from 66 hours to 7 days. The toxin of one specimen of pea juice withstood the heating process for 45 minutes, while in another less vicious product, the destruction of the poison was practically completed in 15 minutes.

The data show, furthermore, that the variation in heat resistance is independent of the original potency of the toxin. Some preparations were more potent than others, but were, nevertheless, less resistant to heat. For instance, spinach samples 3 and 6 (MLD 1:10,000) were less resistant to a temperature of 80 C. than spinach 5 (MLD less than 1:1,000). Similarly asparagus (sample A-1) of relatively high toxicity (MLD 1:10,000) was less resistant than another [sample A-12 (MLD 1:100-1:1,000)] heated at the same temperature. On the other hand, the heat resistance of the most toxic vegetable juices showed at times a relatively high resistance. Spinach 8 and 12 (MLD 1:10,000) were both resistant to a temperature of 80 C., while the toxin content of the most resistant sample of peas (P-1) exceeded 1:1,000,000 MLD; the less resistant one, P-2, contained 100,000 MLD. Therefore no definite relationship can be said to exist between the potency, as expressed by the MLD content of a food juice and the resistance of the toxin to heat. Orr⁴ came to the same conclusions after testing the heat resistance of botulinus toxins produced in peptone broth.

Comparative tests on whole and centrifugalized liquors suggest that whole liquors subjected to a temperature of 80 C. retain their ability to produce botulism in mice or guinea-pigs, although the soluble toxin has apparently been destroyed. It will be noted in chart 3 that centrifugalized asparagus liquor (A-1) resisted a temperature of 80 C. for from 4 to 6 minutes, while the whole specimen heated for 50 minutes, regularly produced botulism in guinea-pigs. The toxicity of one sample of spinach (Sp.-6) when centrifugalized, resisted 12 minutes' heating at 80 C., while the whole liquor still produced botulism in guinea-pigs after maintenance at a temperature of 80 C. for 35 minutes. However, the contents of the tube held at the same temperature for 45 minutes produced symptoms on the 4th day after injection followed by an apparently complete recovery (table 1).

When testing the thermic resistance of the toxin contained in vegetable liquors contaminated with viable spores of *B. botulinus*, the following variables must be considered: (*a*) the effectiveness of 30 minutes' centrifugalization at high speed, (*b*) the influence of the viscosity on the efficacy of this procedure, (*c*) the possible variability in the resistance of the toxin produced in different substrata, (*d*) the virulence of spores of *B. botulinus* for mice and guinea-pigs and (*e*) the influence of foreign substances on the pathogenicity of spores of *B. botulinus*.

TABLE 1

THE PATHOGENICITY OF TOXIC WHOLE AND CENTRIFUGALIZED SPINACH LIQUOR SUBJECTED TO A TEMPERATURE OF 80 C.

Time Heated, Minutes	Spinach 3 Whole Liquor M L D > 1:10,000		Spinach 3 Centrifugalized		Spinach 6 Whole Liquor M L D 1:10,000		Spinach 6 Centrifugalized		Spinach 11 Whole Liquor M L D 1:100-1:1,000		Spinach 11 Centrifugalized	
	Tube	Death of Guinea-Pig	Tube	Death of Guinea-Pig	Tube	Death of Guinea-Pig	Tube	Death of Guinea-Pig	Tube	Death of Guinea-Pig	Tube	Death of Guinea-Pig
2	1	18 hrs.	2	18 hrs.	3	18 hrs.	4	18 hrs.	5	24 hrs.	6	18 hrs.
4	7	40 hrs.	8	24 hrs.	9	24 hrs.	10	24 hrs.	11	72 hrs.	12	80 hrs.
6	13	72 hrs.	14	6 days	15	72 hrs.	16	40 hrs.	17	5 days	18	5 days
8	19	72 hrs.	20	Surv.*	21	78 hrs.	22	72 hrs.	23	8 days	24	7 days
10	25	5 days	26	9 days	27	72 hrs.	28	8 days	29	Surv.	30	Symptoms 5 days surv.
12	31	90 hrs.	32	Surv.	33	72 hrs.	34	11 days	35	8 days	36	Slight sympt. 6 days surv.
15	37	90 hrs.	38	Surv.	39	96 hrs.	40	Surv.	41	Surv.	42	Surv.
20	43	5 days	44	Surv.	45	90 hrs.	46	Surv.	47	Surv.	48	Surv.
25	49	90 hrs.	50	Surv.	51	78 hrs.	52	Slight sympt. 52 hrs. surv.	53	Surv.	54	Surv.
35	55	5 days	56	Surv.	57	5 days	58	Tube broke	59	Surv.	60	Surv.
45	61	5 days	62	Surv.	63	Sympt. 90 hrs. surv.	64	Surv.	65	Surv.	66	Surv.

* Survived.

A few comparative tests on filtered and on unfiltered but centrifugalized broth cultures and toxic spinach liquors showed that in all instances the thermal death time of the toxin was the same before and after passage through the filter. However, in the particular tests quoted, both filtered and unfiltered toxins resisted only 4 minutes' heating. Whether the same conclusions could be drawn if more resistant toxins had been tested in the same manner, cannot be stated at this time. Apparently in such liquors the number of spores left in the clear supernatant fluid after centrifugalization at high speed for 30 minutes

was too few to cause an infection. However, for the more viscous liquors, such as corn and peas, it is quite reasonable to assume that the spores are not completely sedimented by a 30 minutes centrifugalization at high speed. A part of the contents of one sample of corn (size 2½ can) experimentally inoculated with *B. botulinus* strain 97A and incubated at 35 C. for 2 days and at room temperature for 22 days, was centrifugalized at high speed for 30 minutes. The clear liquor was pipetted into another tube and recentrifugalized for a few minutes to remove a few particles that had been carried over from the first tube. After about 5 minutes' centrifugalization, the supernatant was removed and heated in a water bath held at an average temperature of 78 C. After 35 minutes' heating at this temperature, dilutions were made in 5% glucose peptone beef heart broth, covered with a layer of sterile petrolatum and incubated at 35 C. After 2 days' incubation, the first 5 dilutions showed growth. The others remained sterile and remained so during the period of observation, which lasted 6 weeks. The clear heated liquor therefore contained between 100,000 and 500,000 viable spores of *B. botulinus*. Unless the addition of vegetable material has increased their infectivity, it appears rather unlikely that the number of spores retained in the macroscopically clear supernatant fluid is sufficiently large to cause an infection with subsequent intoxication. Furthermore, one would have to consider the spores of *B. botulinus* much more invasive than has generally been established experimentally by several workers. The results are, therefore, either due to an unusually heat resistant toxin or to an increased infectivity of the spores. In the interpretation of the data, it therefore becomes necessary to distinguish between an intoxication and an infection.

Little is known regarding the infectivity of botulinus spores to mice. Experiments presented graphically in chart 3 show that botulism may develop in mice as the result of the introduction of spores heated at 80 C. The results secured in one series of tests conducted with contaminated string bean juice (chart 3, sample SB 12) are particularly striking. In the experiment the centrifugalized liquor was toxic after heating for from 12 to 15 minutes at 80 C., while the samples exposed for a longer period of time were innocuous. The whole liquor, however, was still capable of producing botulism even after 60 minutes' exposure to 80 C. These and similar observations leave no doubt that mice are susceptible to an infection with detoxified botulinus spores suspended in vegetable liquors.

That botulism may be produced in guinea-pigs by the subcutaneous inoculation of detoxified spores, has frequently been noted. However, workers in this field do not agree on the number of detoxified spores necessary to produce an infection in laboratory animals. Coleman and Meyer²³ have shown that in order to produce an infection in guinea-pigs by the subcutaneous route, a relatively large number of detoxified spores must be inoculated. On the other hand, Orr²⁴ and Edmondson, Giltner and Thom²⁵ were able to produce the disease by the subcutaneous introduction of smaller doses. The first workers could produce symptoms quite regularly by the injection of 200,000,000 spores subcutaneously, while in the experiments presented by Orr, 50,000,000 spores were usually sufficient to produce the same result. Edmondson, Giltner and Thom, in one instance produced the disease by inoculating only 30,000,000 spores. Coleman and Meyer are of the opinion that the spores of *B. botulinus* can germinate and multiply in rabbits and guinea-pigs. They demonstrated the presence of vegetative bacilli and toxin in the anterior chamber of the eye and the ligated jugular vein inoculated with detoxified spores. Hall and Davis²⁶ were not successful in producing infections. They believe that the conclusions of Coleman and Meyer are based on insufficient evidence, without, however, presenting experimental data to support this statement. In a later paper, Coleman²⁷ showed that the spores of *B. botulinus*, when enclosed in collodion sacs and placed in the abdominal cavity of guinea-pigs or rabbits, germinate and grow luxuriantly. Additional experimental studies which leave no doubt that *B. botulinus* spores can germinate in the body of laboratory animals will be published from this laboratory in the near future.

Smears made from the toxic vegetable liquors show that the spores represent approximately 30% or less of the total number of bacterial forms. Cultural numerical estimations made by the dilution method²⁸ on samples 3 and 6 in the experiment previously shown in table 1 indicate that a total of 100,000,000 organisms and spores were present in the unheated sample. Thirty million would therefore be a liberal estimate of the number of viable spores present in 1 c. c. of the unheated material. Unfortunately, counts were not made on the heated cultures,

²³ Jour. Infect. Dis., 1922, 31, p. 622.

²⁴ Ibid., 1922, 30, p. 118.

²⁵ Arch. Int. Med., 1920, 26, p. 357.

²⁶ Jour. Exper. Med., 1923, 37, p. 585.

²⁷ Jour. Infect. Dis., 1923, 33, p. 384.

²⁸ Dozier, C. C.: Jour. Infect. Dis., 1924, 35, p. 105.

and the exact number of the viable spores can only be surmised. The following observations leave no doubt that viable spores have been inoculated with the heated, uncentrifugalized vegetable juice. *B. botulinus* was demonstrated in cultures made from the brain of guinea-pig 17, which died in 5 days; from the brain and liver of guinea-pig 23, which died in 8 days; while the organs of guinea-pig 34, which had succumbed 11 days after an inoculation of the heated, centrifugalized spinach liquor, were negative for *B. botulinus*. Furthermore, the inoculation of the heated, whole culture usually produced marked symptoms much more rapidly than the clear supernatant fluids heated for the same period. It seems probable that the intoxication of the animal in the former instance is intensified by the invasion and germination of the spores in the tissues, and is not merely due to the small amount of thermoresistant preformed toxin. This fact, together with the failure to destroy the last traces of toxin absorbed by the foreign matter, may serve in the explanation of the remarkable heat resistance of some of the vegetable juices. However, as previously stated, the presence of the foreign matter in the inoculum may assist in the production of toxin at the sight of the inoculation and in the dissemination of the spores and bacilli throughout the body of the animal.

The assumption that the infectivity of spores of *B. botulinus* may be increased by suspending the inoculum in unfiltered spinach juice instead of salt solution, was in part confirmed by a series of tests carried out to determine this point. Results of tests on guinea-pigs inoculated with increasing numbers of detoxified but viable botulinus spores (2.5-50 million) suspended in unfiltered normal spinach juice and in physiologic salt solution, respectively, indicated that the presence of foreign matter may increase the infectivity of the spores. The animal inoculated with the largest number of spores suspended in spinach juice died after 9 days, while the one inoculated with the same number of spores suspended in salt solution survived. Another animal inoculated with fewer spores (25 million) suspended in salt solution died after 20 days, but since the necropsy findings were inconclusive, no definite conclusions can be drawn. The results are, however, suggestive. It must be remembered that the inoculum did not contain the usual decomposition products present in undiluted, contaminated spinach juice, and that therefore more striking results could be produced if such a suspension were used. It has been pointed out in previous paragraphs that whole heated liquors are regularly more toxic than the clear supernatants, but that the number of spores present in such heated material

could not be as large as is ordinarily required to produce an infection. The experiment just reported should be confirmed with the liquor from this and other products. Much valuable information could probably be obtained by carrying out a series of comparative feeding and inoculation tests.

In order to prove further that the symptoms produced in the guinea-pigs were due to the specific action of botulinus toxin and not to the introduction of foreign material or to other poisonous substances produced by the growth of the anaerobe in plant products, the following experiment was carried out.

TABLE 2
THE PATHOGENICITY OF BOTULINUS TOXIN IN THE LIQUOR FROM CONTAMINATED HOME
CANNED ASPARAGUS SUBJECTED TO A TEMPERATURE OF 80 C.

Time Heated, Minutes	Asparagus 1, M L D 1:10,000, Centrifugalized and Heated		Asparagus 1, Whole and Heated		Asparagus 1, Whole and Heated, 0.2 c c. Antitoxin Added before Inoculation into Guinea-Pig		Asparagus 1, Whole and Heated, then Centrifugalized		Asparagus 1, Whole and Heated, then Centrifugalized, 0.2 c c. Antitoxin Added before Inoculating into Guinea-Pig	
	Tube	Death of Guinea-Pig in About	Tube	Death of Guinea-Pig in About	Tube	Death of Guinea-Pig in About	Tube	Death of Guinea-Pig in About	Tube	Death of Guinea-Pig in About
2	1	18 hours	2	18 hours	3	Survived	4	18 hours	5	Survived
4	11	78 hours	12	40 hours	13	Survived	14	72 hours	15	Survived
6	21	Survived	22	72 hours	23	Survived	24	12 days	25	Survived
8	31	Survived	32	78 hours	33	11 days	34	Survived	35	Survived
10	41	Survived	42	72 hours	43	12 days	44	Survived	45	Survived
12	51	Survived	52	72 hours	53	Survived	54	Survived	55	Survived
15	61	Survived	62	72 hours	63	Survived	64	Survived	65	Survived
20	71	Survived	72	72 hours	73	Survived	74	16 days±	75	Survived
25	81	Survived	82	72 hours	83	15 days±	84	Survived	85	20 days
36	91	Survived	92	72 hours	93	Tube broken	94	13 days neg. aut.	95	13 days neg. aut.
45	101	Survived	102	72 hours	103	Survived	104	Survived	105	Survived

Sealed tubes containing 2 c.c. amounts of whole or centrifugalized liquor from "Asparagus 1" were heated at 80 C. in the usual manner. Three tubes containing the turbid and one containing the clear liquor were removed from the oil bath after definite intervals of heating. Two of the former were then centrifugalized at high speed. Two-tenths c.c. of type A antitoxin, containing about 175 units per c.c., was added to one of these and to the contents of another tube containing the whole heated vegetable juice. After standing at room temperature for about an hour, the toxicity was tested on guinea-pigs in the usual manner (table 2).

The clear supernatant liquor remained toxic for 4 minutes, while the sample, which was centrifugalized after removal from the oil bath

was still poisonous after heating for 6 minutes at 80 C. On the other hand, animals inoculated with approximately 2 c.c. of the whole liquor maintained at 80 C. for 45 minutes died with typical symptoms of botulism in from 3 to 3½ days. Antitoxin protected the animals in all but two cases, when death, preceded by typical symptoms of the disease, occurred after an interval of from 11 to 12 days following the injection of the mixture. The disease-producing properties of the heated whole culture can probably be attributed to the elaboration of toxin in the body of the animal. Had the pathogenicity of these cultures been due to the presence of a trace of toxin, which withstood the heating process, it would have been neutralized by the antitoxin added to the specimen. It is more reasonable to assume that the number of spores in combination with the decomposition products of the spoiled asparagus inoculated was large enough to initiate an infection after the protective action of the antitoxin had worn off.

According to Bull,²⁹ a passive immunity to *B. welchii* infection of at least 12 days can be conferred to man by a single injection of *B. welchii* antitoxin. In a paper dealing with the rate of disappearance of antitoxin from the circulating blood of rabbits inoculated intravenously with diphtheria antitoxin, Glenny and Hopkins³⁰ have shown that after 12 to 13 days' circulating antitoxin could not be demonstrated in the blood serum of rabbits inoculated intravenously with 750 units of diphtheria antitoxin. A very small amount of botulinus antitoxin as compared to the dose used by the other workers has been employed, but judging from the outcome, the animals were protected against an infection for approximately 12 days.

Summary.—Centrifugalized toxins prepared from cultures of *B. botulinus* in peptone veal infusion broth or in beef heart mediums are destroyed by an exposure to a temperature of 80 C. for from 4 to 10 minutes. The average resistance of the toxins produced by 6 strains of *B. botulinus* is 6 minutes, but most of the poison is destroyed during the first 4 minutes. No difference in the relative resistance of the two toxicologic types has been noted.

Liquors obtained from contaminated home canned or from artificially contaminated commercially canned products are more thermo resistant than the toxins produced in glucose veal peptone broth. The results obtained by subjecting 16 samples of centrifugalized toxic vegetable liquors to 80 C. for varying periods of time may be summarized as

²⁹ Jour. Exper. Med., 1917, 26, p. 603.

³⁰ Jour. Hyg., 1922-23, 21, p. 142.

follows: 3 specimens of toxic asparagus juice resisted from 4 to 10 minutes; one beet liquor retained its toxicity after 10 minutes' heating, while two centrifugalized samples of corn juice subjected to a temperature of 80 C. for from 40 and 60 minutes, respectively, remained toxic. At the same temperature, from 8 to 20 minutes were required to destroy the toxin in spinach juice. One sample of toxic pea juice was rendered inert by 15 minutes' heating, while another was still toxic after it had been heated for 45 minutes. One sample of string bean juice resisted 12 minutes' heating.

Ten whole toxic vegetable liquors when heated at 80 C. have shown consistently a higher resistance than the centrifugalized juices. The whole asparagus liquors, for example, withstood for 50 minutes; one sample of beets and one of string beans resisted for 60 minutes, while the resistance of 6 samples of spinach varied from 12 to 60 minutes, depending in all probability on the number of viable spores left in the heated liquor.

The destruction of the *B. botulinus* toxin in centrifugalized liquors as well as the infectious properties of the whole samples of vegetable juice depend not only on the physical and chemical nature of the product, but also on the degree of contamination with viable spores.

C. THE DESTRUCTION OF THE TOXIC PROPERTIES OF CANNED AND
PRESERVED VEGETABLES CONTAMINATED WITH *B. BOTULINUS*
BY HEATING THEM FOR VARYING PERIODS OF TIME
OVER AN OPEN FLAME

From the experimental evidence presented in the preceding pages, it is obvious that the poison produced in broth cultures is destroyed more readily at a temperature of 80 C. than the toxin produced in vegetables. It was shown that uncentrifugalized vegetable liquors subjected to a temperature of 80 C. may produce botulism when inoculated subcutaneously into guinea-pigs or mice. This property is retained by the liquors for a considerably longer time than is ordinarily necessary to destroy the soluble botulinus toxin. Whether such heated and apparently detoxified material is infectious after ingestion was not determined. One is naturally not justified in drawing general conclusions from the results of test-tube experiments nor in applying them to the practical problems encountered in the household. It was thought advisable to determine the effect of cooking spoiled and toxic vegetable products over an open flame for varying periods of time under conditions similar to those found in any household. Samples of the cooked or heated food were fed to guinea-pigs instead of being inoculated subcutaneously.

Numerous epidemiologic observations [report 4 spinach; 47 asparagus³¹] throw considerable doubt on the repeatedly published statements that suspicious canned goods when emptied into a kettle and boiled for 5 minutes can be eaten without danger. For example, a portion of the content of a spoiled 2½ can of spinach vigorously boiled for 20 minutes proved fatal to a guinea-pig on subcutaneous inoculation, and when fed to the same species of animal caused typical botulism in 3 days, depending on the bulk of the spoiled foods, size of the kettle, and numerous uncontrollable factors. Complete detoxification is usually only accomplished after boiling for at least 30 minutes, depending on the altitude.

The term cooked or "heated" is often misleading. Products, particularly those of thick consistency and badly spoiled, appear to be boiling long before the true boiling point is reached and even before the temperature is sufficiently high to destroy the toxin. The possibility of mistaking the apparent boiling point as the true one was pointed out by Thom.¹⁶ He states, however, that no appreciable time is required to detoxify a product which has reached a temperature of 85 C. This may be true as far as test-tube experiments are concerned, but it is by no means proved for the ordinary household procedure in which the spoiled product is removed from the can or jar and boiled in an open kettle. Here the unequal distribution of heat throughout the mass as well as the possible delay in heat penetration into the pulp of the product influences the detoxification. Burke, Elder and Pischel have pointed out that spoiled canned string beans may appear to be boiling for 7 minutes, and yet the juice may be fatal to guinea-pigs on subcutaneous inoculation. If, experimentally, this is true for string beans, in which the amount of liquor is relatively large and the viscosity low, then one could reasonably expect that products such as corn, peas, or spinach would require a still longer exposure and even higher temperatures to destroy their disease-producing properties. The results of numerous feeding experiments carried out with samples of contaminated beets, corn, peas, spinach, and string beans heated over an open flame for various periods of time confirmed the findings reported in section B, and supplied additional evidence to support the already published data concerning the infectivity of spores of *B. botulinus*. The experiments are detailed in the following pages.

³¹ Pub. Health Bull., 1922, 127.

Methods.—Canned toxic vegetables either naturally or experimentally contaminated with *B. botulinus* were heated slowly over a low gas flame in an open saucepan. Changes in temperature were followed by inserting several thermometers at different levels of the product. In this way, difference in the distribution of heat could be followed. The temperature at which the product seemed to be boiling was recorded as the "apparent boiling point." From time to time, a small portion, about the size of a hen's egg, was taken from the container and placed in a sterile Petri dish. The food was thoroughly stirred after the removal of each sample, and whenever necessary, 50-150 c.c. of hot tap water were added to prevent the burning of the product. About 2 tablespoonfuls of crushed barley were mixed with the cooled sample and fed to guinea-pigs weighing from 225 to 300 gm. Since the animals were not given their usual feeding on the morning of the experiment, the mixture was consumed ordinarily within from 8 to 15 hours; in rare instances when spoiled corn or peas were fed, it became necessary to keep the animals in individual cages for 40 hours before all of the food was consumed, but in such instances variations in the outcome of the tests could not be detected. This difficulty was not encountered in feeding string beans or spinach. After the food was eaten, the animals were placed in larger cages and examined usually twice, and whenever possible three times, daily. Even then symptoms have escaped detection. At times, particularly in those animals dying during the first 48 hours after the ingestion of the heated product, symptoms were of such short duration that they passed unnoticed. According to van Ermengem,¹ very strong doses of toxin may bring forth symptoms which last only $\frac{1}{4}$ to $\frac{1}{2}$ hour in a guinea-pig or rabbit. Bengtson²⁰ reports a case in which a guinea-pig inoculated intraperitoneally with a type A toxin died after 1 hour and 40 minutes without showing symptoms of botulism. In the experiments to be reported, similar observations were made. It was, furthermore, noted that shock or handling may hasten the death of the intoxicated guinea-pig. In one instance, 2 animals fed with heated spinach died within 18 hours. A 3rd animal fed with a sample heated for a shorter period of time failed to show symptoms 20 hours after the ingestion of the food. The guinea-pig was removed to a larger cage and was found dead one hour later, no symptoms having been recorded. In such instances, when typical necropsy findings were noted and secondary infections were absent, the death of the animal was ascribed to botulism produced by the ingestion of the contaminated spoiled product. If the animals died and showed typical lesions at necropsy after a lapse of longer periods of time, although symptoms were not detected, the food fed was considered suspicious. Animals were observed over a period of from 3 to 4 weeks and were then discarded.

THE DESTRUCTION OF BOTULINUS-TOXIN IN ARTIFICIALLY CONTAMINATED CANNED BEETS

The data collected by feeding toxic beets heated over an open flame for varying periods of time are presented in table 3.

The toxic liquors from cans number 1 and 2 were poured off and an equivalent amount of tap water was added. After cutting the beets in small blocks (about 2-3 c.c.) they were placed in a saucepan over a low gas flame and cooked. The rise in temperature throughout the product was comparatively uniform, and at the "apparent boiling point" the temperature was close to 95 C. However, the toxin was destroyed even before the liquor was actively boiling. What effect

the dilution of the poison by the addition of water may have had, cannot be definitely stated. From the experimental data shown in table 3, it seems that the addition of such large amounts of water hastened the destruction of the toxin either by mere dilution or by decreasing the number of spores in the infected product. From the third experiment of this series, it appears that the

TABLE 3
THE PATHOGENICITY OF CANNED BEETS CONTAMINATED WITH *B. BOTULINUS*, AFTER HEATING OVER A LOW GAS FLAME

Sample 1. Inoculated with 10 Billion Heated Spores of <i>B. botulinus</i> (7 Strains) and Incubated for 5½ Months at 35 C.; Poured Off Part of Toxic Liquor and Added 150 c.c. Tap Water before Heating M L D of Toxic Liquor 1:50,000				Sample 2. Inoculated with 10 Billion Heated Spores of <i>B. botulinus</i> (7 Strains) and Incubated for 5½ Months at 35 C.; Poured Off Toxic Liquor and Added 250 c.c. Tap Water before Heating M L D of Toxic Liquor 1:1,444-1:5,000			
Time Heated, Minutes	Temperature, C.	Sample Fed	Death of Guinea-Pig in About	Time Heated, Minutes	Temperature, C.	Sample Fed	Death of Guinea-Pig in About
0	22	1	16 hours	0	22	1	24 hours
3	42-46			2	30-36		
4	52-58			5	52-54	2	24 hours
5*	61	2	24 hours	8	71-74		
6	72-81			10*	83-85	3	24 hours
7	82-84			12†	95		
8†	91-96	3	Lost after 24 days	12½	99-100		
10	97-100			14	99-100	4	Survived
13	101	4	Survived	20	99-100	5	Survived
15	99-100	5	Survived	30	99-100	6	Survived
20	99-100.5	6	Survived	40	99-100	7	Survived
30	99-100.5	7	Survived	50	102	8	Survived
35‡	99-100.5	8	Survived				
45‡	99-100.5	9	Survived				
55	99-100.5	10	Survived				

Sample 3. Inoculated with 17 Billion Heated Dried Spores of <i>B. botulinus</i> (Strains 38A and 97A); Incubated for 55 Days at 35 C.; Weakly Toxic 2 c.c. Killed Guinea-Pig in 16 Hours; 0.1 c.c. negative				Sample 4. Inoculated with 10 Billion Heated Spores of <i>B. botulinus</i> (7 Strains); Incubated for 5½ Months at 35 C.; after Opening Held for About Ten Days at Room Temperature M L D 1:1,000					
Time Heated, Minutes	Temperature, C.	Symptoms of Guinea-Pig	Death of Guinea-Pig in About	Time Heated, Minutes	Temperature, C.	Sample Fed	Death of Guinea-Pig in About	Subcutaneous Inoculation of Supernatant Fluid from 1 Gm. Sample	
								Symptoms	Death
0	29	24 hrs.	0	14	1	18 hrs.		
2	46-48			2	20-70	2	18 hrs.		
4	54-55	18 hrs.	5	3	40 hrs.		
5	60			6*	46-77				
6*	18 hrs.	40 hrs.	7	48-75				
7	89-82			8†	50	4	40 hrs.		
8†	89-95	40 hrs.±	10	58-87	5	40 hrs.	40 hrs.	48 hrs.
10	99-97			11	72-91				
11	100-99	Lost	13	80-95	6	40 hrs.	48 hrs.
15	100	48 hrs.	15	91-95	7	Survived	5 days	9 days
20	100	40 hrs.	48 hrs.	20	92-98	8	19 days	Surv.
26	100	Survived	23§	93-94				
30	100	Survived	30	94-98	9	Survived	Surv.
				34§	100				
				45	100	10	6 days	Surv.

* Gas bubbles appear throughout mass.

† Apparent boiling point.

‡ 50 c.c. hot tap water added.

§ 100 c.c. hot tap water added.

toxicity of the product is less readily destroyed when the toxic supernatant fluid is not removed. Although the potency of the toxin in the liquor from this tin of beets was low, guinea-pigs fed with samples heated for 15 and 20 minutes developed botulism. The temperature had been above 80 C. for 8 and 13 minutes, respectively, but the beets proved to be toxic, when fed.

Another tin of toxic beets was kept at room temperature for several days after the can was opened. They became slightly disintegrated, and the liquor was heavier than usual. In this instance, it should be noted that no water was added prior to heating. On account of the consistency of the product, the rise in temperature was somewhat delayed and more irregular than in the less viscous food. After 8 minutes' heating, although the temperature recorded at this time was only 50 C., the apparent boiling point was reached. Consequently, the resistance of the toxin appeared to be greater than in the first two experiments. In addition to the usual feeding tests as carried out in the foregoing experiments, 1 gm. each of samples 5-10 was ground in 2 c.c. of sterile salt solution, and after 30 minutes' centrifugalization at high speed, the supernatant fluid was inoculated subcutaneously into guinea-pigs weighing from 250 to 300 gm. It will be noted from table 3 that a weak toxin could be demonstrated in a sample that had been apparently boiling for 7 minutes. The temperature had been above 80 C. for at least 2 minutes. The toxin in the beets although weakened had not been entirely destroyed by the heating process. Another animal fed with a whole sample heated for 20 minutes died in 19 days. No symptoms were recorded, but a typical picture of botulism was revealed at necropsy.

Summary.—The contents of 4 cans of commercially canned beets artificially contaminated with several strains of *B. botulinus* and incubated at 35 C. were heated over an open flame, and from time to time were tested for toxicity by feeding small amounts of the heated product to guinea-pigs. The pathogenic properties of the contents from 2 cans from which the poisonous liquor had been removed and an equal volume of water added are destroyed as soon as the temperature has risen above 85 C. The beets to which no water is added must be heated at least for from 7 to 20 minutes above 80 C. before the poison is destroyed. The addition of water to the unheated vegetable facilitates the destruction of the poisonous properties of the spoiled product.

EFFECT OF HEAT ON TOXIC PROPERTIES OF CANNED CORN AND PEAS CONTAMINATED WITH *B. BOTULINUS*

From the experimental data presented in table 4 it appears that the destruction of the toxic substances liberated by the growth of *B. botulinus* in canned corn and peas is not accomplished in so short a period of time as might be expected.

After from 3 to 10 minutes' heating, the product seemed to be boiling, although the temperature was far below 100 C. Both foods, but particularly corn, appeared to be boiling for a considerable period of time without reaching a temperature sufficiently high to destroy the

TABLE 4

THE PATHOGENICITY OF SPOILED CANNED CORN AND PEAS CONTAMINATED WITH *B. BOTULINUS*, AFTER HEATING OVER A LOW GAS FLAME

Sample 1, Commercially Canned Corn Inoculated with 17 Billion dried Spores of Strains 38A and 97A Heated at 80 C. for 1 Hour; Incubated at 35 C. for 4 Days, Room Temperature for 52 Days M L D 1:100,000					Sample 2, Commercially Canned Corn Inoculated with 100 Million Heated Spores of Strains 38A, 54A and 62A; Incubated at Room Temperature for 10 Months; Badly Spoiled M L D 1:1,000-1:10,000			
Time Heated, Minutes	Temperature, C.	Sample Fed	Symptoms Noted	Death of Guinea-Pig in About	Time Heated, Minutes	Temperature, C.	Sample Fed	Death of Guinea-Pig in About
0	17	1	18 hrs.	0	15	1	18 hrs.
2	31-60				2†	32-72	2	18 hrs.
3*	36-68	2	18 hrs.	3†	45-58		
3½†	45-70				5	72-80	3	18 hrs.
5	32-84	3	18 hrs.	6	86-92		
6	75-90				7	98-97	4	Lost
7	83-90	4	24 hrs.	40 hrs.	8	94-98		
8	83-91				10	95-99	5	Survived
10	97	5	66 hrs.	14	6	Survived
13	100	6	72 hrs.	96 hrs.	17	96-98	7	72 hrs.
16	100	7	10 days±	20	8	Survived
20	100	8	13	15 days slight pneumonia	21†			
					25	88-95	9	Survived
					32	97	10	Survived
					40	98-99	11	Survived
					41§			
					50	98-99	12	Survived
					60	98-99	13	Survived

Sample 3, Jar of Corn Obtained from Yakima, Washington; Corn Dry, Little Evidence of Gas Formation; Butyric Acid Odor; 150 c.c. Tap Water Added before Heating. M L D 1:100					Sample 4, Commercially Canned Corn Inoculated with 1 Billion Spores of Strain 97A Heated at 100 C. for 10 Minutes; Incubated at 35 C. for 10 Days and at Room Temperature for 9 Days; Badly spoiled				
Time Heated, Minutes	Temperature, C.	Sample Fed	Symptoms Noted	Death of Guinea-Pig in About	Time Heated, Minutes	Temperature, C.	Sample Fed	Symptoms Noted	Death of Guinea-Pig in About
0	1	24 hrs.	0	18	1	18 hrs.
2	28-37				2	21-80			
3*	20-58				4	25-75			
4	27-80				5*	2	18 hrs.
5	2	24 hrs.	28 hrs.	5½†	74			
6†	67-91				6½†	72-90			
8	73-93				8	85-98	3	7 days slight pneumonia
10	93-96	3	72 hrs.	96 hrs.					
15	96-99	4	6 days	7 days					
20	5	6 days	7 days	10	97-99			
23†	99	6	4 days	4 days	13	100	4	40 hrs.
30	98	7	18 days sick not Bot.	19 days pneumonia	19	100	5	40 hrs.
					25	100	6		
					33	100	7		
35	98	8	6 days neg. aut.	40	100	8	40 hrs.
40†	98	9	72 hrs.	96 hrs.	40	100	9	11 days	5 days± 12 days
45	98	10	Survived					
50	98	11	40 hrs.	48 hrs. ? aut.					
60	98	12	Survived					

Sample 1, Commercially Canned Peas Inoculated with 100 Million Heated Spores of Strains 38A, 54A; Incubated at Room Temperature for 10 Months; Badly spoiled M L D 1:100,000					Sample 2, Commercially Canned Peas Inoculated with 17 Billion Spores of Strains 38A and 97A Heated at 80 C. for 1 Hour; Incubated at 35 C. for 4 Days and at Room Temperature for 9 Days; Badly Spoiled				
---	--	--	--	--	---	--	--	--	--

TABLE 4—Continued

Time Heated, Minutes	Temperature, C.	Sample Fed	Symptoms Noted	Death of Guinea-Pig in About	Subcutaneous Inoculation of Supernatant Fluid from 1 Gm. Sample	Time Heated, Minutes	Temperature, C.	Sample Fed	Symptoms Noted	Death of Guinea-Pig in About
0	15	1	18 hrs.		0	15	1	18 hrs.
2	21-31					4	48-55			
5	37-42	2	18 hrs.		5	48-61	2	18 hrs.
8	74-81	3	18 hrs.		7	71			
9†	78-85					8	78-80	3	18 hrs.
10	82-90	4	5 days	6½ days		9*	88-91			
11	89-93					10†	98-100	4	40 hrs.
13	89-98	5	Surv.	Survived	11	98-100			
16	98-99	6	40 hrs.	48 hrs.	Survived	12	98-100	5	40 hrs.
20§	7	48 hrs.	21 days neg. aut.	15	98-100	6	66 hrs.	80 hrs.
						20	98-100	7	5 days	5 days
21	80-91					25‡	98-100	8	5 days	5 days
23½	87-93	8	24 hrs.	48 hrs.	Survived	30‡	98-100	9	5 days	7 days
30§	99	9	5 days	6 days	Survived	35‡	98-100	10	8 days	9 days
40	99	10	Surv.	Survived	40	98-100	11	7 days
47†										neg. aut.
50	99	11	Surv.	19 days neg. aut.	45	98-100	12	18 hrs. neg. aut.
51§										
60	99	12	Surv.	Survived					

* Gas bubbles appear throughout mass.

† Apparent boiling point.

‡ 50 c.c. hot tap water added.

§ 100 c.c. hot tap water added.

toxin. It will be noted that corn was never detoxified at the apparent boiling point. Likewise, one sample of peas that had reached a temperature of 98-100 C. at the apparent boiling point proved to be toxic when fed to guinea-pigs. Even prolonged heating does not detoxify the product. It will be noted that 96 hours after ingesting a small portion of corn cooked for 13 minutes, a guinea-pig died with typical symptoms of botulism. In the same experiment, another developed symptoms 13 days after eating corn heated for 20 minutes, although in this case the temperature had been above 90 C. for at least 10 minutes. The toxin produced in a second can of corn was destroyed in a shorter period of time (7 minutes), while in the 3rd and 4th samples, 40 minutes' heating was insufficient to make the product safe for consumption. The results in the last experiment (sample 4) were slightly irregular. Guinea-pigs 8 and 9 were fed a portion of the same sample of corn heated for 40 minutes. One died within 5 days after partaking of the food, but no symptoms were noted. The other developed typical symptoms of the disease 11 days after eating the food and died on the 12th day. The irregularities in these tests were in all probability due to differences in the number of spores ingested.

Exactly similar results were noted after heating canned peas artificially contaminated with *B. botulinus*. The contents of the first

can was heated and sampled in the usual way. In addition, 1 gm. samples of the collections 5-10 were ground in 2 c.c. of sterile salt solution, centrifugalized, and the clear supernatant fluids inoculated subcutaneously into guinea-pigs. Although toxin could not be demonstrated by the latter method, guinea-pigs fed with the usual amount of samples and 6, 7, 8 and 9 developed symptoms of the disease and died in from 2 to 6 days. Since free toxin was no longer present, the production of botulism must be attributed to an infection rather than an intoxication. Samples from the contents of a second can heated for even a longer period of time was still capable of producing the disease. The temperature had been above 90 C. for at least 15 minutes in the first product and for 25 minutes in the second.

Summary.—The contents of 3 cans of corn, artificially contaminated with *B. botulinus* and that of 1 jar of toxic home canned corn, heated over an open gas flame for from 17 to 50 minutes, during which time the temperature was above 80 C. for from 9 to 42 minutes, respectively, are capable of producing botulism in guinea-pigs. The contents from artificially contaminated canned peas similarly tested retain their disease-producing properties when cooked for from 30 to 35 minutes or exposed to a temperature above 80 C. for from 20 to 22 minutes. One experiment indicates that although the toxin itself is destroyed, the ingestion of the contaminated heated product is still capable of producing botulism in guinea-pigs.

THE DESTRUCTION OF BOTULINUS TOXIN IN CONTAMINATED CANNED SPINACH

Four specimens of canned or preserved spinach contaminated with *B. botulinus* were heated in the usual manner (table 5).

The first can tested had been seized at Yakima, Wash., and sent to the laboratory for examination. About 250 c.c. of the slightly disintegrated spinach was placed in an enamel cooking pot over a low gas flame. A thermometer inserted through the cover was immersed just far enough to cover the mercury bulb, a 2d one was placed through another small opening and kept in contact with the sides of the container, while a 3rd was kept in the middle of the saucepan. In this experiment, the samples were removed after thoroughly mixing the contents. It will be seen from this and subsequent tests that, although the food was frequently stirred, the distribution of heat was very uneven. After 6 minutes heating, the potency of the toxin had not decreased materially. The specimen removed 2 minutes later was less toxic; the temperature recorded at this time fluctuated from 78-98 C. Symptoms in the guinea-pigs fed with subsequent samples (6, 8 and 9) were not recorded, but necropsy findings were typical for botulism, while the guinea-pig fed with sample No. 7 survived.

In a second specimen heated, 1 gm. portions of samples 5 to 14 were emulsified in sterile salt solution, centrifugalized as previously described and injected subcutaneously into guinea-pigs. The ordinary feeding tests were also made. The temperature had reached only 79 C. at the apparent boiling point, but the presence of toxin could be demonstrated by both methods. Five minutes later, or after a total of 16 minutes' heating, toxin was still present, although the food

TABLE 5

THE PATHOGENICITY OF SPOILED CANNED SPINACH CONTAMINATED WITH *B. BOTULINUS*,
AFTER HEATING OVER A LOW GAS FLAME

Sample 1. Can of Toxic Spinach seized at Yakima, Wash.; Slightly Disintegrated M L D 1:10,000				Sample 2. Commercially Canned Spinach Artificially Contaminated with 10 Billion Heated Spores of <i>B. botulinus</i> (7 Strains); Incubated at 35 C. for 5½ Months M L D 1:5,000				
Time Heated, Minutes	Temperature, C.	Sample Fed	Death of Guinea-Pig in About	Time Heated, Minutes	Temperature, C.	Sample Fed	Death of Guinea-Pig in About	Supernatant Fluid, 1 Gm. Sample
0	20	1	18 hrs.	0	17	1	18 hrs.	
2*	30-55	2	18 hrs.	3	17-46	2	21 hrs.	
3	30-42			5*	32-43			
4	39-82	3	18 hrs.	6	35-71	3	18 hrs.	
5	49-78			8	80-93			
6	55-95	4	18 hrs.	9	76-95	4	18 hrs.	
7	78-98			11†	79	5	40 hrs.	22 hrs.
8	79-95	5	5 days	13	79-93			
9	85-99	6	29 days	14	6	40 hrs.	5 days
10	93-100	7	Survived	16	79-97	7	Survived	7 days
12	91-99			17½	95-99	8	Survived	Survived
15‡	95-99	8	16 days	22	100	9	Survived	Survived
30	99	9	16 days	25	98-96	10	Survived	Survived
				30	98	11	Survived	Survived
				32‡				
				37	98	12	Survived	Survived
				42§				
				45	98	13	Survived	Survived
				48§				
				60	98	14	Survived	Survived

Sample 3. Commercially Canned Spinach Artificially Contaminated with 17 Billion Spores of <i>B. botulinus</i> (Strains 38A and 97A Heated at 80 C. for 1 Hour); Incubated at 35 C. for 54 Days M L D 1:1,000					Sample 4. Commercially Canned Spinach Artificially Contaminated with 17 Billion Spores of <i>B. botulinus</i> (Strains 38A and 97A Heated at 80 C. for 1 Hour); Incubated at 35 C. for 67 Days M L D 1:10,000				
Time Heated, Minutes	Temperature, C.	Sample Fed	Symptoms Noted After	Death of Guinea-Pig in About	Time Heated, Minutes	Temperature, C.	Sample Fed	Symptoms Noted After	Death of Guinea-Pig in About
0	29	1	16 hrs.	0	25	1	18 hrs.
2*	42-71	2	16 hrs.	3*	28-75			
3½†	45-60				5†	34-95	2	18 hrs.
5	55-80	3	16 hrs.	7	51-97			
7	78				8	57-93			
8	87-84	4	16 hrs.	10	87-96	3	18 hrs.
11	84-90	5	3 days	5 days	15	86-98	4	72 hrs.	90 hrs.
15	89-99	6	9 days±	20	90-97	5	Survived
16	95-96				25‡	83-99	6	48 hrs.	60 hrs.
20	99-100	7	72 hrs.	80 hrs.	27	87-96			
25‡	8	Survived	30	98-97	7	48 hrs.
30‡	97-100	9	72 hrs.	80 hrs.	35‡	90-97	8	Survived
36	95-99	10	48 hrs.	41	9	27 days
				? aut.	45‡	98-97	10	Survived
					55	98-97	11	Survived
				48 hrs.	60	98-97	12	6 days
42‡				— aut.					
45	11						

TABLE 5—Continued

Sample 5, Commercially Canned Spinach Artificially Contaminated with 17 Billion Spores of <i>B. botulinus</i> (Strains 38A and 97A Heated at 80 C. for 1 Hour); Incubated at 35 C. for 41 Days. M L D about 1:500					
Time Heated, Minutes	Temperature, C.	Sample Fed	Death of Guinea-Pig in About	Supernatant Fluid from 1 Gm. Sample Subcutaneously Inoculated	
				Symptoms	Death in About
0	20	1	18 hrs.		
2	22-55				
5	24-92	2	18 hrs.		
6	54				
10	71-96	3	18 hrs.		
12	78-96				
14	86-96	4	Survived		
16	94-98				
18	5	Survived	3 days
22	98	6	7 days	6½ days	7 days
26	98	7	7 days	7 days
30	99	8	4 days, neg. aut.	7 days
35	99	9	7 days	7 days, neg. aut.
40	99	10	8 days salvation	Survived

* Gas bubbles appear throughout mass.

† Apparent boiling point.

‡ 50 c.c. hot tap water added.

§ 100 c.c. hot tap water added.

had been stirred several times in the interval elapsing between the two tests. The temperature recorded varied from 79 to 97 C. Apparently several portions of the spinach had not yet reached a temperature sufficiently high to destroy the poison. However, longer heating entirely destroyed the toxic properties of the contaminated food.

The next two experiments show, however, that botulism may occur after the ingestion of apparently thoroughly boiled spinach. It will be seen in table 5 that typical symptoms of the disease developed within 3 days after the ingestion of spinach heated for 30 minutes, although the temperature had been above 80 C. for about 22 minutes in the 1st, and 20 minutes in the 2d case. A sample removed after 41 minutes heating proved fatal to a guinea-pig in 27 days. No symptoms had been reported, but the lesions revealed at necropsy seemed typical of the disease. It cannot be definitely stated, however, that this animal had succumbed of botulism, but the sample must be considered as suspicious.

It may be stated without hesitancy that the destruction of the toxic properties of canned spinach contaminated with *B. botulinus* is met with great difficulty. In some samples, probably in those less extensively infected, its destruction is completed in a comparatively short time, and no fatalities result from the ingestion of the detoxified food. In others, at least 20 to 22 minutes' heating above 80 C. is necessary to destroy the disease-producing properties of the contaminated product. Esty and Meyer³² have shown that the destruction of spores subjected to a temperature of 100 C. is a gradual process. In their tests, which were carried out in a buffered phosphate solution, approxi-

³² Jour. Infect. Dis., 1922, 31, p. 650.

mately 1,000,000 of 10,000,000,000 spores survived heating at 100 C. for 30 minutes, e. g., the number of spores was decreased ten thousand-fold. It is probable that the final destruction of the pathogenic properties of the contaminated food is due to the killing off of the less resistant spores by exposure to a temperature of 100 C. for a considerable length of time.

THE DESTRUCTION OF BOTULINUS-TOXIN IN STRING BEANS

The results obtained by heating one can and one jar of toxic string beans in the usual manner may be briefly summarized as follows:

After 1½ minutes' heating at a temperature ranging from 98 to 99 C., the sample from the weakly toxic contents of the first container (MLD 0.1 c.c.; weekly toxic) was still slightly poisonous to guinea-pigs. Two and one-half minutes later the poison had been completely destroyed. The contents of the second jar submitted to the test (MLD 1:100) were markedly decomposed. On account of the disintegration of the pods, the heat penetration into the pulp was reduced to a minimum. A temperature of 60 to 68 C. for a short period of time was sufficient to reduce the potency of the toxin, as evidenced by the delayed appearance of symptoms in a guinea-pig fed with the warmed product. However, a temperature ranging from 82 to 85 C. for 1½ minutes was sufficient to destroy the poison. From the experimental data, it appears that the toxic properties of contaminated string beans can be destroyed by subjecting them to a temperature above 85 degrees for 5 minutes. We are unable to state whether string beans containing more potent toxins and possibly more highly contaminated with spores are rendered harmless in so short a period of time. Burke, Elder and Pischel³ found that 19 minutes' exposure to temperatures constantly approaching 100 C. was necessary to detoxify poisonous string bean juice completely. However, their specimens were inoculated subcutaneously, and as they suggest, the intoxication was probably due to the injection of living spores. The series reported in this paper is not sufficiently large to rule out the factors of varying degree of contamination and toxicity.

The observations recorded in this paper leave no doubt that the detoxification of vegetable products containing botulinus toxin requires more time and higher temperatures than is generally supposed to be the case. Although it is frankly admitted that the products used in the experiments were so badly decomposed that most of the housewives would have discarded them, it is known that one sample of asparagus

(A-1, A-3) and one of corn (Yakima) came from botulism outbreaks (reports 90 and 107). The dogmatic assertions of certain workers that decomposed food is resurrected by boiling, is therefore refuted by epidemiologic observations. In fact, just these observations were responsible for the studies presented in the preceding paragraphs. Furthermore, the policy recommended by a number of food officials to educate the consumer of canned products to recook the food preserved in glass or tin before it is eaten, has obviously its limitations. This is particularly true, when the advice must reach and be appreciated by people who are not only careless in the selection of the raw products, and their processing, but also in judging spoilage of canned goods and in deciding the degree of heat or the time of boiling necessary to render it safe.

The experiments recorded in this paper indicate that the botulinus toxin present in vegetable liquors is more rapidly destroyed by diluting the content of the jar or can with an equal amount of water before heating. A common practice to prevent the burning of the food serves therefore as an excellent safeguard against botulism poisoning and should be generally advocated. It should, however, be remembered that recooking may well reduce the number of fatal human cases, but it may fail to render the food harmless for domesticated animals that have access to partially cooked material. The only protection against botulism remains therefore in the proper education of the public to use adequately processed food products, whether commercially or home canned. Visibly spoiled canned food should not be made safe by recooking. It should be destroyed by adding to the jar a fair amount of commercial lye, which is commonly used in the household. The heat generated will not only destroy the botulinus toxin, but will in conjunction with the high degree of alkalinity either destroy or materially reduce in number the potentially dangerous spores of *B. botulinus* present in the food.

CONCLUSIONS

The toxin produced by the growth of *B. botulinus* in broth culture mediums and in vegetable products may be destroyed by the prolonged action of direct sun, diffuse daylight, and air. Unfiltered but centrifugalized toxic asparagus liquor or broth cultures resist about 90 to 118 hours' exposure to the action of direct sunlight and air. When the poison is kept anaerobically, it remains toxic for a longer period of time.

Exposure of broth toxins or of toxic vegetable products to the action of diffuse daylight and air at room temperature causes a slow

but progressive loss of potency. A decrease in the strength of the liquor is detected after 2½ months' exposure. Exposure to air hastens the destruction of the poison.

Vegetable toxins kept in the dark on ice retain their potency or show only a slight loss in strength over a period of from 5 to 8 months.

Fourteen tests on the toxins produced by the growth of 6 strains of *B. botulinus* in glucose peptone veal broth and in beef heart mediums and maintained at a temperature of 80 C. for varying periods of time, showed that the poison is destroyed in about 6 minutes. Individual toxins may vary in resistance, but no difference exists in the resistance of the two toxicologic types.

The poison produced by the growth of the organism in vegetables is usually more resistant to exposure to a temperature of 80 C. than the toxin produced in culture mediums.

The resistance of toxins formed in canned vegetables may vary in different or even in the same product.

The variation is independent of the original potency of the poison.

Whole liquors are regularly more resistant than the centrifugalized specimens and are capable of producing botulism after the soluble toxin has apparently been destroyed.

The destruction of the poison in contaminated toxic vegetables, such as beets, corn, peas, spinach, and string beans, by cooking over an open flame is difficult and uncertain. Such products may appear to be boiling long before a temperature sufficiently high is reached to destroy the toxin. This observation confirms the results of other workers.

In products such as beets and string beans in which the heat penetration is rapid, maintenance at a temperature above 80 C. for 2½ minutes may be sufficient to destroy the toxicity of the product.

In highly contaminated and markedly spoiled products, such as peas and corn, or in those in which the heat penetration is slow, as in spinach, maintenance at a temperature rising steadily from 80 C. to 100 C. for an average of 21 minutes was not sufficient to destroy the disease-producing properties of the food. It is suggested that the pathogenic properties of such heated food is due to the ingestion of spores which have survived the heating process rather than a failure to destroy the free toxin in the infected food.

Suspicious food although thoroughly cooked is not fit for human or animal consumption.

FORMATION OF PEROXIDE BY ACTINOMYCES NECROPHORUS ON EXPOSURE TO AIR IN RELATION TO ANAEROBIC PLATE CULTURES

WILLIAM A. HAGAN

*From the Department of Comparative Pathology and Bacteriology, New York State
Veterinary College, Cornell University, Ithaca*

THE PRODUCTION OF PEROXIDE BY CULTURES OF AN OBLIGATORY ANAEROBE
(ACTINOMYCES NECROPHORUS) WHEN EXPOSED TO AIR

McLeod and Gordon¹ showed that the inhibitory substance which appeared in cultures of pneumococcus, and which had been called "bactericidin" by McLeod and Govenlock² was, in reality, hydrogen peroxide. A number of coccus forms, in addition to the pneumococcus, were shown to be peroxide formers when grown in the presence of air. Callow,³ investigating the question of whether or not the sensitivity of anaerobic bacteria to air could be explained on the basis of peroxide formation coupled with a lack of ability to form catalase, found that all of the obligatory anaerobes with which she worked were devoid of catalase, but she was unable to obtain proof that any of them were capable of forming peroxide.

McLeod and Gordon⁴ obtained a greenish zone in "shake" cultures of several anaerobes about $\frac{1}{8}$ inch beneath the surface in cooked blood agar. Since a similar color was obtained in cultures of several aerobic organisms which were known to be peroxide formers, and was not obtained in the case of any aerobe which did not produce peroxide, they regarded this phenomenon as an indication of peroxide formation. They did not obtain any direct chemical evidence of peroxide formation by anaerobes other than the action on blood, and the significance of this reaction may be open to question.

Working on the hypothesis that it was the accumulation of peroxide in cultures of anaerobic organisms, made possible by a lack of catalase, which was responsible for failure of these organisms to grow in the presence of the free oxygen of the air, Avery and Morgan⁵ showed

Received for publication June 9, 1924.

¹ Biochem. Jour., 1922, 16, p. 499.

² Lancet, 1921, 1, p. 900.

³ Jour. Path. and Bacteriol., 1923, 26, p. 320.

⁴ Ibid., 1923, 26, p. 332.

⁵ Jour. Exper. Med., 1924, 39, p. 289.

that several obligate anaerobes could be grown in plain broth to which had been added some small bits of raw, sterile potato. When boiled or autoclaved, the potato lost most of its growth promoting function, indicating that this function depended on some heat labile substance, probably of enzyme nature. Coincident with the loss of its growth promoting function, the potato lost its catalase and possibly other peroxidases. Since the same authors had just shown that the oxidase content of a bit of raw potato prevented the formation of hydrogen peroxide in fluid cultures of pneumococcus grown in presence of air,⁶ they thought it probable that it was in this way that the potato functioned in promoting growth of the anaerobes under aerobic conditions. Avery and Morgan⁷ also were able to show that when small amounts of an artificial peroxidase (an iron salt) was added to plain broth, several anaerobes could be cultivated through repeated transfers in the presence of air, thus apparently settling the question of whether or not anaerobic bacteria can be grown under aerobic conditions if only a supply of the oxidases are furnished in the medium.

Avery and Morgan apparently accepted, without question, McLeod and Gordon's evidence for the ability of anaerobes to form peroxide when exposed to air, and their own work has greatly strengthened the circumstantial evidence leading to this conclusion. In their publications, however, I have not seen any record of an attempt to prove directly that H_2O_2 was produced by anaerobic bacteria.

When difficulty was experienced in obtaining growth of *Actinomyces necrophorus* after exposure to air (see part 2), the thought occurred that peroxide formation might explain these results. Accordingly, vigorous young cultures of the organism grown in plain broth and in cooked meat medium⁸ under a petrolatum seal were poured into Petri dishes and flasks in shallow layers and thus exposed to the air. Periodic tests for peroxide formation were made, using a saturated solution of benzidine in glacial acetic acid as the reagent and a bit of raw potato to furnish the necessary oxidases, as suggested by Avery and Morgan.⁶ These authors state that the benzidine test is not specific for hydrogen peroxide, and that a reaction would probably be obtained from any peroxide which the peroxidases of the plant tissue were capable of splitting. However, it has been proved by McLeod and Gordon¹ that

⁶ Ibid., p. 275.

⁷ Proc. Soc. Exper. Biol. and Med., 1923, 21, p. 59.

⁸ Finely ground, fat-free beef is cooked for one hour with twice its weight of water to which has been added 2% peptone and 0.5% NaCl. Normal alkali is added until the supernatant fluid after thorough stirring gives an orange red color to phenol red. It is tubed, covered with a layer of petrolatum and autoclaved for one hour at 15 lbs.

the peroxide formed by the pneumococcus was really hydrogen peroxide, and there is considerable evidence in this paper to indicate that the peroxide formed by this anaerobe is also the peroxide of hydrogen. The results of the tests are given in table 1.

TABLE 1
PRODUCTION OF PEROXIDE BY CULTURES OF ACTINOMYCES NECROPHORUS INCUBATED UNDER PETROLATUM WHEN EXPOSED TO THE AIR IN SHALLOW LAYERS, AS DETERMINED BY THE BENZIDINE TEST

Time of Test	Plain Broth 24-Hour Culture	24-Hour Cooked Meat Medium Culture	
		Supernatant Fluid Without Meat Fragments	Supernatant Fluid With Some Fragments of Meat
Immediate.....	—	—	—
1 hour.....	+	—	—
	Faint		
2 hours.....	+	—	—
	Strong		
3 hours.....	+	+	—
		Faint	
4 hours.....	+	+	+
		Strong	Faint
6 hours.....	+	+	+
			Stronger*
8 hours.....	+	+	+
			Much weaker
10 hours.....	+	+	—
	Weaker	Weaker	
17 hours.....	+	Not tested	Not tested
	Faint		

* This reaction not so strong as that in fluid without meat fragments.

Repeated tests show that in plain broth cultures, a positive peroxide test generally can be obtained in 2 hours or less after exposure to air. The supernatant fluid from the cooked meat cultures usually gave positive peroxide tests in from 2 to 3 hours when no gross meat fragments were present, but when meat fragments were present, the reaction was delayed, diminished in intensity and sometimes failed to develop. In fluids without meat fragments, the peroxide reaction could be obtained for some hours, although the intensity of the reaction soon began to diminish. In the presence of meat fragments, on the other hand, if peroxide developed in a concentration sufficiently great to give a positive test to benzidine, it quickly disappeared. Repeated tests indicate that the heat sterilized meat fragments possess the ability to destroy peroxide, and the question of whether a positive reaction develops or not in fluids containing such fragments depends on whether peroxide is formed faster than it is destroyed. All of the tests indicated that, under the conditions permitted, young, active cultures of *Actinomyces necrophorus* accumulated peroxide actively for from 4 to 6 hours after

exposure to the air. In the presence of meat fragments, peroxide destruction goes on fairly rapidly, so that when the peak of active production has been passed, the fluid soon gives a negative test.

Table 2 illustrates the ability of sterile plain infusion broth and cooked meat medium to destroy hydrogen peroxide. When sufficient titrated hydrogen peroxide was added to each medium to make a dilution of 1:10,000, a strong benzidine test was obtained immediately. Within three hours, however, the meat medium had destroyed so much of the peroxide that a positive test could barely be obtained, and later the test became completely negative. In the plain broth, the intensity of the benzidine reaction was practically unchanged after 24 hours.

TABLE 2
THE PEROXIDE SPLITTING ABILITY OF STERILE MEDIUMS *

Time Tested	Plain Infusion Broth	Cooked Meat Medium
Immediately.....	Strongly +	Strongly +
1 hour.....	Strongly +	Strongly + but weaker than before
2 hours.....	Strongly +	Weakly +
3 hours.....	Strongly +	Trace
4 hours.....	Strongly +	Negative
24 hours.....	Strongly + Intensity of reaction diminished very little if any	Negative

* Titrated hydrogen peroxide was added to sterile mediums in amounts sufficient to make a dilution of 1:10,000. The results of the benzidine test at intervals afterward are given.

The peroxidase-like action of sterile meat medium was shown in another manner. When 1 or 2 c.c. of a 3% solution of H_2O_2 was pipetted into the medium through a melted petrolatum seal, and the seal quickly hardened by holding the tube in cold water, a small bubble of gas always formed beneath the seal after a few minutes. The introduction of a solution of KOH did not diminish the size of the bubble, but it was completely absorbed when a little pyrogallol was introduced with the KOH. This indicates that the gas was oxygen, and that the peroxide was being broken up. Hydrogen peroxide added to plain broth and to the centrifugalized supernatant fluid of sterile cooked meat medium did not give rise to gas formation.

The substance in meat which is responsible for the destruction of hydrogen peroxide is not known—possibly it is the iron residue of broken down hemoglobin. Since this medium was cooked for several hours and finally sterilized by autoclaving at about 120 C. for one hour, it is hardly possible for catalase, or ordinary organic peroxidases, to survive, since these are heat labile substances.

The Amount of Peroxide Produced by Actinomyces Necrophorus.—Assuming the peroxide formed by this organism to be hydrogen peroxide, a rough idea of the concentration accumulated by cultures exposed to the air was gained by making up a series of dilutions of hydrogen peroxide in the sterile medium and comparing the intensity of the benzidine reactions in these to those obtained in the cultures. It was found that in plain broth, the test would detect readily as little as 1 part of H_2O_2 in 100,000 parts of fluid, and occasionally 1 in 200,000.⁹ The reactions obtained in cultures when at the height of their intensity, i. e., after 4 or 5 hours' exposure, generally were very much stronger than those obtained in a H_2O_2 dilution of 1:100,000. Many of them approached but hardly reached the intensity given by H_2O_2 in 1:10,000 dilution.

The Inhibitory Action of H_2O_2 for Actinomyces Necrophorus.—To test the inhibitory action of known concentrations of H_2O_2 on this organism, sufficient of the peroxide was added to plain broth and to cooked meat medium under petrolatum seal to make concentrations of 1:10,000 and 1:100,000, these being amounts comparable to that produced naturally by the cultures on exposure to the air. Each tube was inoculated heavily (0.5 c.c.) and incubated until growth appeared, or it became evident that the culture would not develop (table 3).

TABLE 3
THE INHIBITING ACTION ON ACTINOMYCES NECROPHORUS OF SEVERAL DILUTIONS OF HYDROGEN PEROXIDE IN PLAIN BROTH, AND IN COOKED MEAT MEDIUM; 0.5 C.C. OF A 48-HOUR PLAIN BROTH CULTURE USED AS THE INOCULUM. EACH SYMBOL REPRESENTS GROWTH OR ABSENCE OF GROWTH IN ONE CULTURE

Concentration of H_2O_2	Cooked Meat Medium 24 Hours	Plain Broth		
		24 Hours	48 Hours	120 Hours
0 Control.....	++	-----	++++
1:10,000.....	++	-----	-----	-----
1:100,000.....	++	-----	++++

As was to be expected, all of the cooked meat cultures grew promptly. Later tests were made when as much as 1:1,000 concentration of peroxide in meat medium was inoculated, and growth obtained, although the meat fragments showed visible signs of the bleaching effect of the peroxide. In plain broth, a 1:10,000 dilution of peroxide

⁹ Avery and Morgan claim that they were able to detect peroxide in dilutions only as high as 0.002%, i. e., 1:50,000 (Jour. Exper. Med., 1924, 39, p. 347).

completely inhibited growth, while a 1:100,000 dilution showed some lag, although this was no greater than that exhibited by the control.

The Bactericidal Action of H₂O₂ for Actinomyces Necrophorus.—Hydrogen peroxide to form dilutions of 1:10,000 and 1:100,000 was added aseptically to vigorous cultures growing in plain broth, and at intervals tubes of cooked meat medium were inoculated with massive doses (0.5 c.c.), since it was known that the meat medium would quickly destroy the peroxide carried over. The results are incorporated in table 4.

TABLE 4

HYDROGEN PEROXIDE WAS INTRODUCED INTO VIGOROUS 48-HOUR CULTURES OF ACTINOMYCES NECROPHORUS IN PLAIN BROTH. AT INTERVALS SUBCULTURES (0.5 c.c.) WERE MADE INTO COOKED MEAT MEDIUM. EACH SYMBOL REPRESENTS GROWTH OR ABSENCE OF GROWTH IN ONE SUBCULTURE

Hydrogen Peroxide, 1:10,000						
Time of Exposure to H ₂ O ₂	16 Hours	24 Hours	36 Hours	48 Hours	72 Hours	96 Hours
Not exposed.....	++
1 hour.....	—	—	+-	++
2 hours.....	—	—	+-	++
3 hours.....	—	—	+-	++
4 hours.....	—	—	—	+-	++
5 hours.....	—	—	—	—	—	+-
6 hours.....	—	—	—	—	—	—
Hydrogen Peroxide, 1:100,000						
All exposures up to 4 hours.....	++
4 hours.....	—	++
5 hours.....	—	—	++
6 hours.....	—	—	++

It is to be noted that hydrogen peroxide in a dilution of 1:100,000 showed some inhibition, but all cultures were growing at 36 hours. In 1:10,000 dilution, however, a marked period of lag was evident in the subcultures made after short exposures to the peroxide solution, and some of the later subcultures failed to grow, indicating that at this concentration hydrogen peroxide is distinctly injurious, and, if the exposure is long enough, even bactericidal.

The Relation Between Peroxide Formation and the Lag Periods which Occur in Cultures of Actinomyces Necrophorus Transplants which Have Been Exposed to Air.—When cultures of Actinomyces necrophorus were exposed to the air, subcultures made into plain mediums generally failed to develop, and those into the more favorable

cooked meat medium showed a pronounced period of lag, which varied directly, within certain limits, with the duration of the period of the exposure. It has been shown above that a similar lag period was evident in cultures to which had been added hydrogen peroxide to form a concentration about the same as was formed by cultures exposed to the air. It is also significant that the time of accumulation of appreciable amounts of peroxide by cultures coincides closely to the periods in which the bacterial lag begins in the subcultures. The correlation between lag periods and peroxide formation is illustrated by table 5.

TABLE 5

SUPERNATANT FLUID OF A 24-HOUR COOKED MEAT MEDIUM CULTURE OF ACTINOMYCES NECROPHORUS EXPOSED TO THE AIR IN A SHALLOW LAYER, AND SUBCULTURES MADE AT INTERVALS INTO FRESH COOKED MEAT. EACH SYMBOL REPRESENTS GROWTH OR ABSENCE OF GROWTH IN ONE SUBCULTURE; INOCULUM, 1 LOOP

Time of Exposure	Test for H_2O_2	Subcultures			
		24 Hours	48 Hours	72 Hours	96 Hours
Not exposed.....	—	++
½ hour.....	—	++
1 hour.....	—	++
2 hours.....	—	++
3 hours.....	—	—	++
4 hours.....	+ Faint	—	—+	++
5 hours.....	+ Strong	—	—+	—+	—+
6 hours.....	+ Strong	—	—	—+	—+
8 hours.....	+ Weaker	—	—	++
Control culture in tube, 8 hours.....	—	++

The evidence that the lag periods in cultures exposed to air is due to the accumulation of hydrogen peroxide is as follows:

(a) The lag periods begin at the time when an appreciable amount of peroxide has accumulated in the culture fluid.

(b) The length of the lag periods increases as the amount of peroxide increases.

(c) After peroxide accumulation reaches its height and the amount begins to decrease, the length of the lag periods does not increase further.

(d) The addition of hydrogen peroxide to cultures, in amounts approximating that found in cultures exposed to air, produces lag periods in the subcultures similar to those observed in subcultures from cultures exposed to air.

THE REASON FOR FAILURE TO OBTAIN GROWTH OF ACTINOMYCES NECROPHORUS ON
PLATE CULTURES INCUBATED IN AN ANAEROBIC JAR

Mohler and Morse¹⁰ reported difficulty in inducing *Bacillus* (*Actinomyces*) *necrophorus* to grow in plate cultures incubated anaerobically, although they were successful in cultivating it on the same medium in other ways. They do not discuss the cause of this difficulty.

Recently, in attempting to plate out some cultures of this organism, we experienced the same difficulty. In "hormone" agar (Huntoon¹¹) "shakes," good, though not vigorous, growth was easily obtained, but when the same medium was inoculated, poured into plates and incubated in a Brown anaerobic jar,¹² failure to obtain growth nearly always resulted.

In preparing the plate cultures, the inoculated medium necessarily is exposed to the air from the time it is poured into the plates until anaerobic conditions become established in the jar. Since the ratio of the exposed surface to the total volume of the medium is large, there is opportunity for the dissolving of a considerable volume of atmospheric oxygen, and it was thought that in this fact the cause of the failures might lie.

To test this hypothesis, fluid cultures were exposed to the air in shallow layers in Petri dishes and flasks, and subcultures made at intervals into cooked meat medium. During the exposures, the fluid was protected from the light. Some representative results are given in tables 6 and 7.

Although growth generally was obtained in the transplants from cultures exposed to the air, a striking period of bacterial lag resulted, the duration of which varied directly with the time of exposure up to 3 or 4 hours, beyond which little difference was noted in the behavior of the subcultures; whereas 0.1 c.c. or even 1 loopful of normal vigorous 24-hour culture sufficed to give vigorous growth with marked gas production in from 18 to 24 hours in cooked meat medium, after exposure to the air from 1 to 4 hours the subcultures required from 48 to 96 hours to show evidence of growth, and some failed altogether. In several instances, cultures were exposed for periods up to 8 hours, but no appreciable difference was noted in the subcultures made after 3 or 4 hours. The reason for this will become apparent later.

¹⁰ U. S. Dept. Agric., Bu. Animal Industry Bulletin 167, 1905.

¹¹ Jour. Infec. Dis., 1918, 23, p. 169.

¹² Jour. Exper. Med., 1921, 33, p. 677.

Cooked meat medium is the most favorable substrate for the cultivation of *Actinomyces necrophorus* which we have found. It is far superior to the plating mediums which we have used in encouraging and supporting growth and in addition it is peculiarly fitted for correcting abnormal oxidation reduction balances. When subcultures were made from cultures exposed to the air into plain infusion broth under petrolatum seal, a medium more nearly like the plating mediums than is cooked meat, it was found that growth occurred only occasionally when the exposure was as long as 30 minutes and as much as 0.1 c.c. was used for the inoculum. In longer exposures, growth failure was nearly invariable.

TABLE 6

SUPERNATANT FLUID OF A 48-HOUR COOKED MEAT MEDIUM CULTURE OF *ACTINOMYCES NECROPHORUS* EXPOSED TO THE AIR IN A SHALLOW LAYER, AND SUBCULTURES MADE AT INTERVALS INTO FRESH COOKED MEAT. EACH SYMBOL REPRESENTS GROWTH OR ABSENCE OF GROWTH IN ONE SUBCULTURE; INOCULUM, 0.1 c.c.

Time of Exposure	Subcultures			
	24 Hours	48 Hours	72 Hours	96 Hours
Not exposed.....	÷ + + +
15 minutes.....	+ + + +
1 hour.....	+ - - -	+ + + +
2 hours.....	- - - -	- + + +	+ + + +
4 hours.....	- - - -	- - - -	- + + +	+ + + +

TABLE 7

CULTURE IN PLAIN BROTH OF *ACTINOMYCES NECROPHORUS* INCUBATED UNDER PETROLATUM SEAL FOR 24 HOURS, THEN EXPOSED TO THE AIR IN A SHALLOW LAYER AND SUBCULTURES MADE AT INTERVALS INTO COOKED MEAT. EACH SYMBOL REPRESENTS GROWTH, OR ABSENCE OF GROWTH, IN ONE SUBCULTURE; INOCULUM, 0.1 c.c.

Time of Exposure	Subcultures				
	24 Hours	48 Hours	72 Hours	96 Hours	144 Hours
Not exposed.....	+ +
1 hour.....	- -	- -	+ +
2 hours.....	- -	- -	+ -	+ -	+ -
4 hours.....	- -	- -	- -	- -	- -
6 hours.....	- -	- -	- -	+ +

The hypothesis that exposure to the air was the cause of the plating failures now seemed likely to be correct. To prove this directly, means of minimizing the exposure of the medium to the air was sought. Two methods were tried and both proved successful. Briefly, these were:

(a) Plates of "hormone" agar, using about 30 c.c. instead of the customary 10 to 12 c.c. per plate, were poured. With as little delay as possible they were

placed in the jar and anaerobic conditions established.¹³ Good growth was obtained after 72 hours' incubation. All of the colonies, however, were located in a zone about 2 mm. thick next to the bottom of the plate. No colonies developed within 5 or 6 mm. of the surface. Plates containing about 12 c.c. of the same agar, inoculated with the same quantity of the same culture and incubated in the same jar, failed to show growth.

(b) Plates of hormone agar were poured and the surfaces flooded with sterile liquefied petrolatum as quickly as the agar congealed. After incubation in the anaerobic jar, good growth was obtained. Plates without the petrolatum in the same jar did not show growth. Plates with the petrolatum, incubated aerobically also failed.

Discussion.—The data indicate that the failure to obtain growth of *Actinomyces necrophorus*, when plated anaerobically, was due to injury of the bacterial cells forming the inoculum, by reason of their contact with air, so they were unable to multiply in a medium which was not highly favorable but which, nevertheless, supported growth readily from uninjured inocula of equal size.

That contact with air, even though of short duration, was injurious to the vegetative forms of strict anaerobes, was first pointed out by Pasteur¹⁴ in 1861 in his communication on the butyric acid ferment. When he passed a current of CO₂ through the liquid in which the butyric acid vibrio was multiplying, life and reproduction was not affected, but if a stream of air was introduced for one or two hours, the organisms perished and the fermentation ceased.

More recently, Bachman¹⁵ showed that a large proportion, and sometimes all, of the vegetative forms of several anaerobes lost their vitality when exposed to the air for periods as short as 10 to 30 minutes. Barber,¹⁶ in attempting to establish pure line strains of a series of anaerobes, found that the vegetative forms were not nearly so satisfactory as spores for seeding material. In 301 isolations of vegetative cells, he succeeded in initiating growth in only 42 instances in subculture (1:7), whereas dealing with the spores of the same organisms, growth in subcultures occurred in 93 instances of 211 isolations (1:2). Great differences in the viability of the vegetative forms of different organisms were noted. Barber attributes the difference in the results when spores and vegetative cells were used to the effect on the latter of the exposure to air. In his technic, the exposure lasted only a few minutes.

¹³ The operation of the jar was controlled by the growth of *C. botulinum* on other plates in the jar, and by the complete reduction of an aqueous solution of methylene blue in alkaline dextrose gelatin.

¹⁴ Compt. rend. Acad. d. Sc., 1861, 52, p. 344.

¹⁵ Centralbl. f. Bakteriöl., 1912, 36, p. 1.

¹⁶ Jour. Exper. Med., 1920, 32, p. 295.

The data given brings out the fact that when plating anaerobic bacteria the exposure of the medium to the air, in some cases at least, sufficiently injures the vegetative forms to delay and even to prevent growth. Data in the accompanying paper indicate that this injury is due to the formation and accumulation of hydrogen peroxide in the medium.

SUMMARY

It has been shown that an obligate anaerobe, *Actinomyces necrophorus*, produces sufficient peroxide, when young, vigorous cultures are exposed to the air in shallow layers to give a relatively strong test to benzidine and fresh potato. Peroxide appears to accumulate for 4 to 6 hours, after which its rate of production diminishes or ceases.

The peroxide rather quickly disappears after formation, especially when meat fragments are present in the culture fluid. It has been shown that the meat fragments contain some heat resisting substance in small amounts which operates like a peroxidase.

Judging by the intensity of the reaction given to benzidine, cultures of this organism produce nearly 0.01% of peroxide (1:10,000) calculated as hydrogen peroxide, when exposed to the air, in medium which is free from active peroxide-destroying power.

When hydrogen peroxide was added to young, vigorous cultures of *Actinomyces necrophorus* to make a dilution of 1:10,000, subcultures behaved much like those made from cultures which had been exposed to the air. Since it was estimated that the concentration of peroxide which accumulates in cultures exposed to air was about 1 part in 10,000, this is taken as evidence that the peroxide formed is actually peroxide of hydrogen and not organic peroxide. The instability of the peroxide formed by the culture is also suggestive of hydrogen peroxide.

The periods of bacterial lag in subcultures made from cultures of *Actinomyces necrophorus* which have been exposed to air, are due to the accumulation of peroxide, probably hydrogen peroxide, in the culture fluid.

Actinomyces necrophorus usually failed to develop on plate cultures incubated anaerobically, although it developed readily in "shake" cultures in the same medium. The failure to grow on plate cultures incubated anaerobically was shown to be due to cell injury resulting from exposure to the atmosphere while the medium was being poured and hardened before being placed in the anaerobic jar.

A LYTIC PRINCIPLE (BACTERIOPHAGE) FOR CORYNEBACTERIUM DIPHTHERIAE

JOHN E. BLAIR

From the Department of Biology of Brown University, Providence

In discussing the bacterial species for which a lytic principle has been isolated, d'Herelle¹ makes the following statement:

Two strains of bacteriophage active for only atoxic strains of Bact. diphtheriae have been isolated from the feces of two horses immunized by the injection of cultures of diphtheria bacilli. This observation is only mentioned. Lack of opportunity has prevented further examination of these strains, a study of which certainly offers much of interest.

Botez² claimed to have obtained lysis in series using diphtheria, pseudodiphtheria, dysentery and anthrax bacilli, by introducing into broth cultures of these organisms one loopful of a saturated alcoholic solution of methyl violet.

The work now described was undertaken in the attempt to isolate a lytic principle against *Corynebacterium diphtheriae* from guinea-pigs: (a) from the intestine by d'Herelle's technic, and (b) from the peritoneal exudate by the method of Bordet and Ciuca; also from normal cultures of the diphtheria organism by allowing the cultures to age.

ISOLATION OF A LYTIC PRINCIPLE BY D'HERELLE'S METHOD

The technic of the experiments follows:

A small amount of fecal material, about 4 mm. in diameter, was emulsified in 20 c.c. of broth, and incubated at 37.5 C. for 18 hours. Dunham's peptone broth, with a reaction of P_H 8.0-8.2 was used. After incubation, this culture was diluted with three volumes of sterile broth, and filtered. A preliminary filtration through filter paper, or centrifuging, was followed by filtration through a Mandler filter, no. 4. The filtrate thus obtained was tested for the presence of the bacteriophage. In this testing, enough filtrate was added to a broth suspension of the test organism to give a dilution of 1:5. The presence of a bacteriophage was shown by lysis of the organisms and a clearing up of the culture.

A guinea-pig was injected intraperitoneally with a suspension of a young blood serum culture of slightly toxic diphtheria bacilli. On the 4th day, the animal was killed and examined. There was the characteristic area of congestion at the point of inoculation, and the internal organs showed typical, although not extreme, signs of diphtheria intoxication. From 3 levels of the

Received for publication, June 2, 1924.

¹ Bacteriophage, 1922.

² Compt. rend. Soc. de biol., 1921, 85, p. 585.

small intestine and from the colon, material was removed and emulsified in sterile broth. After being treated according to d'Herelle's method, as described, the filtrate was tested against 4 strains of diphtheria organisms. It was inactive for 3 strains, and dissolved one strain; this strain was atoxic.

Another guinea-pig was inoculated subcutaneously with a toxic strain of *C. diphtheriae*, and died in about 40 hours. Typical pathologic changes were found at necropsy. A small amount of the contents of the small and large intestine was removed between 3 and 4 hours after the animal died. The material was treated according to d'Herelle's method. The filtrate was slightly active against 7 strains of diphtheria bacilli, and showed no activity against 3 strains. Three of the strains partly dissolved were toxic; all other strains tested were atoxic. No lysis of the homologous strain was produced.

Three stools of a child convalescing from diphtheria were examined. The filtrates varied in their ability to produce lysis. One was active against its homologous strain and also against 7 others (including 3 toxic strains), and dissolved 3 strains only slightly. The other 2 filtrates were from stools taken on 2 successive days. One of these filtrates showed no activity. The other dissolved 5 strains, was weakly active for 3, and had no effect on 3 others.

ISOLATION OF A LYTIC PRINCIPLE BY THE METHOD OF BORDET AND CIUCA

Bordet and Ciuca³ were able to demonstrate the presence of a lytic principle active against *Bact. coli* in the peritoneal exudate of guinea-pigs that had received inoculations of the organism at intervals of a few days. Others⁴ repeated the experiment, using other organisms.

The technic of Bordet and Ciuca was modified slightly for these experiments. Immediately after the guinea-pigs were killed, the peritoneal exudate was removed and diluted with 5 volumes of sterile broth. After 18 hours' incubation, the mixture was filtered through a Mandler filter, no. 4, and the filtrate tested.

A guinea-pig was inoculated intraperitoneally with a suspension of a young culture of diphtheria bacilli of low virulence. After 90 hours, the guinea-pig was killed, and about 1 c.c. of exudate removed. The exudate was diluted with 10 volumes of broth instead of 5, treated as outlined, and the filtrate was tested against 4 strains of diphtheria bacilli. It dissolved 2 of the strains, and showed no activity against the other 2. The filtrate from the intestinal contents of the same animal dissolved only 1 of the 4 strains.

A highly virulent strain of *C. diphtheriae* was inoculated subcutaneously into a guinea-pig, killing it in 40 hours; 5 c.c. of exudate were removed from the peritoneal cavity, and treated according to the

³ Ibid., 1920, 83, p. 1293.

⁴ Wollstein: Jour. Exper. Med., 1921, 34, p. 467. Bail: Wien. klin. Wchnschr., 1921, 34, p. 447. Gratia and Jaumain: Compt. rend. Soc. de biol., 1921, 85, p. 880. Kuttner: Jour. Bacteriol., 1923, 8, p. 49.

method described. Two strains of diphtheria were dissolved by the filtrate, and 9 strains were untouched. A filtrate from the intestinal contents showed slight activity for 7 strains, and did not affect 3.

A guinea-pig was inoculated intraperitoneally with a suspension of a throat culture containing diphtheria bacilli in almost pure culture. After 21 hours, the animal showed decided symptoms of intoxication, and was killed. Necropsy showed a typical picture of diphtheria intoxication. Approximately 12-14 c.c. of exudate were removed and treated as above. The filtrate was markedly active for 2 strains, dissolved 6 others weakly, and was inactive for 3.

ISOLATION OF A LYTIC PRINCIPLE FROM BACTERIAL CULTURES

Several investigators⁵ have reported the isolation of lytic substances from cultures of bacteria of varying ages. The cultures have been from 18 hours to 1 or 2 months old in some cases.

An attempt was made to isolate a lytic principle for *C. diphtheriae* from old cultures. A diphtheria culture on blood serum was kept at room temperature for 2 months. At the end of this time the growth was washed off in sterile broth, diluted to 30 c.c., and filtered through a Mandler filter. When tested against 4 strains of diphtheria, the filtrate showed only a weak activity. A broth culture of another strain of diphtheria was allowed to remain at room temperature for 35 days. It was then filtered through a Mandler filter. When tested against a subculture of the same strain, the filtrate was inactive. Neither did it produce lysis of 3 other strains of diphtheria bacilli.

SPECIFICITY OF THE LYTIC PRINCIPLE

Certain of the filtrates obtained in my experiments were tested against members of the colon-typhoid-dysentery group, to determine their specificity. Many of them that dissolved diphtheria strains, were also active in varying degrees for colon, typhoid or dysentery bacilli. Lysis was confirmed in the case of these organisms by the appearance of "plaques" or the formation of lytic colonies on agar.

A filtrate obtained from the peritoneal exudate of a guinea-pig that died from *Bact. coli* peritonitis caused lysis of 1 of 4 strains of *C. diphtheriae*. It also dissolved colon, typhoid, and dysentery bacilli. Antityphoid bacteriophages, obtained from stools of a typhoid con-

⁵ Bail: *Wien. klin. Wchnschr.*, 1921, 34, p. 555. Pico: *Compt. rend. Soc. de biol.*, 1922, 87, p. 833. Weinberg and Aznar: *Ibid.*, 1922, 86, p. 833.

valescent, had no effect on 4 diphtheria strains. They produced lysis of one or more members of the colon-typhoid-dysentery group.

SEPARATION OF CULTURES INTO SENSITIVE AND RESISTANT STRAINS

After exposure for 6 days to the action of a filtrate, a diphtheria culture was streaked on an agar plate. Two distinct types of colonies developed. Both had depressed centers; one type was entirely translucent, while the raised edges of the other type were opaque. When exposed to the homologous filtrate, subcultures of the translucent colonies did not grow; organisms from the opaque-edged colonies grew discretely. Hence the organisms composing the translucent colonies might be termed "sensitive," and those of the second type of colony "resistant."

In diphtheria cultures that had been exposed to the action of a saturated alcoholic solution of methyl violet, Botez² noticed some club-shaped forms, as well as some granular and short forms. On microscopic examination, the translucent colonies I have described were found to consist almost entirely of coccoid and swollen forms, only about 5% being of the small, more regular type often found in agar cultures of diphtheria. The translucent centers of the second type of colony contained similar forms, while the opaque, raised edges were composed almost entirely of small regular forms. Similar colonies containing organisms of similar morphology were produced from 3 strains of diphtheria.

Neither the sensitive nor the resistant strains caused the death of 250 gm. guinea-pigs when suspensions were introduced subcutaneously in 2 c c. amounts. The parent strain killed a 250 gm. guinea-pig in 40 hours.

PROTECTIVE VALUE OF THE LYTIC PRINCIPLE

Experiments have been described^{1, 6} which show that a lytic principle may have some protective value, or, in some cases, therapeutic value. A 24-hour culture of a toxic strain of *C. diphtheriae* was suspended in 2 c c. of sterile broth. The suspension was diluted with an equal volume of a filtrate which had previously been found to be active against that strain; 2 c c. of the mixture were then injected subcutaneously into a

⁶ Kabeshima: *Compt. rend Acad. d. sc.*, 1919, 169, p. 1089. Bruynoghe and Maisin: *Compt. rend Soc. de biol.*, 1922, 86, p. 294. Gratia: *Ibid.*, 1922, 86, p. 276.

guinea-pig weighing 250 gm. The guinea-pig did not die in 4 days. Another guinea-pig of the same weight, inoculated subcutaneously with 2 c.c. of a suspension of the same strain of diphtheria alone, died in 40 hours. A third guinea-pig, inoculated with 2 c.c. of a mixture of

TONSIL AND ADENOID CLINIC, 1921

School Reports	No. Operations	Improved Mentally	Improved Physically
Hillside.....	32	5	32
Open-air.....	19	2	17
1.....	6	0	0
2.....	18	2	2
3.....	167	105	134
4.....	127	30	20
5 and annex.....	147	85	146
6.....	122	61	3
7.....	101	66	81
8.....	100	30	63
9.....	312	228	272
10.....	201	153	183
11.....	106	20	24
12.....	102	39	72
13.....	122	27	38
15.....	78	22	26
16.....	171	121	136
17.....	277	109	170
18.....	342	213	290
19.....	128	67	108
20.....	90	31	50
21.....	58	3	5
22.....	298	165	204
23.....	14	1	2
24.....	139	79	115
25.....	89 (1921)	86	86
25.....	39 (1922)	17	17
26.....	257 (1921)	162	252
26.....	78 (1922)	16	53
27.....	787	228	492
28.....	104	17	20
29.....	21	21	21
30.....	91 (1921)	66	50
30.....	9 (1922)	7	2
31.....	128	12	11
32.....	59	22	51
33.....	126	95	107
34.....	55	6	6
35.....	30	7	5
36.....	63	57	63
37.....	110	49	49
38.....	34	4	11
39.....		No report	
40.....	63	37	48
41.....	85	39	57
42.....	16	16	16
43.....	51	35	46
44.....	86	52	79
Totals.....	5,658	2,715	3,735

equal parts of the filtrate and sterile broth, was not affected. This result confirms the reports of the majority of investigators, viz., that a lytic principle has a definite protective value, and also that the lytic principle in itself is not harmful to the animal.

SUMMARY

A lytic principle active against *Corynebacterium diphtheriae* was isolated (*a*) from the intestinal contents of guinea-pigs inoculated with diphtheria bacilli, (*b*) from the peritoneal exudate of guinea-pigs inoculated with diphtheria bacilli, (*c*) from a diphtheria culture two months old.

Two strains of diphtheria bacilli possessing different degrees of resistance were isolated by the action of a lytic principle on a virulent strain. These 2 strains were not virulent for guinea-pigs weighing 250 gm.

The majority of the lytic principles obtained were not specific. They dissolved several strains of diphtheria bacilli, as well as members of the colon-typhoid-dysentery group.

A guinea-pig inoculated with a mixture of equal parts of a lytic principle and a suspension of diphtheria bacilli was not killed in 4 days. A guinea-pig inoculated with the same strain of diphtheria bacilli alone died in 40 hours.

OCCURRENCE OF ORGANISMS OF THE ENTERITIDIS PARATYPHOID B GROUP IN GUINEA-PIGS

BERNARD G. H. THOMAS*

From the Pathological Laboratories, University of Pittsburgh, Pittsburgh

I. BACTERIOLOGICAL STUDIES

The prevalence of infections among laboratory animals is a common observation among those doing experimental work. The extent of these infections may be gaged by the recent studies of Lynch,¹ Topley,² and Tenbroeck³ on white mice, and of Holman,⁴ O'Brien,⁵ Krumwiede,⁶ and Howell and Schultz⁷ on guinea-pigs. Although the organisms recovered by various workers have been wide in range, including *Streptococcus*, *Staphylococcus*, *B. bronchisepticus* and *pneumococcus*, and other less important types, those giving rise to epizootics have largely been identified as inhabitants of the intestinal tract and belong to the enteritidis-paratyphoid group. The readiness with which animals may be infected enterally by feeding these organisms has been demonstrated by Amoss⁸ and by Petrie and O'Brien.⁵ Under adverse environmental conditions, these bacteria may become widely disseminated throughout the animal body, giving rise to foci of inflammation of varying intensity in different organs. The object of the present studies is to emphasize the importance of these organisms in giving rise to spontaneous lesions in animals used for experimental work.

During the fall of 1922, an attempt was made to produce a streptococcic glomerulonephritis in guinea-pigs. It was observed that the kidneys of both the treated and the supposedly normal control animals showed pathologic lesions which were similar and varied in degree only. Some days later there occurred a period of cold weather, following which many of the stock animals died, and the kidney lesions found in the latter animals were also identical with those in the experimental ones.

Received for publication, June 18, 1924.

* R. B. Mellon Fellow in Pathology.

¹ Jour. Exper. Med., 1922, 36, p. 15.

² Jour. Hyg., 1921, 20, p. 103.

³ Jour. Exper. Med., 1920, 32, pp. 19, 33.

⁴ Jour. Med. Res., 1916, 35, p. 151.

⁵ Jour. Hyg., 1910, 10, pp. 231, 287.

⁶ Jour. Med. Res., 1919, 39, p. 449.

⁷ Jour. Infect. Dis., 1922, 30, p. 516.

⁸ Jour. Exper. Med., 1922, 36, p. 25.

Organisms of the enteritidis paratyphoid B group were recovered from several guinea-pigs that died spontaneously, and this suggested the advisability of determining, if possible, whether such organisms were the causative agents of the pathologic changes observed or were merely contributory factors.

In the present study are recorded the results of the bacteriologic findings in 47 guinea-pigs. Twenty-seven of these animals died of spontaneous infections, 12 received experimental injections, and 8 were killed as controls. Rabbits were used in the production of immune serum, and in the determination of the virulence of certain of the paratyphoid organisms isolated from the guinea-pigs.

Cultures were made throughout from heart blood and spleen, and occasionally also from lung and liver, as soon after the death of the animal as possible to avoid postmortem bacterial invasion. Of the 27 animals that died spontaneously, 7 gave negative cultures. From the remaining 20 animals, organisms giving the cultural characteristics of the enteritidis animal paratyphoid B group were obtained 6 times; nonhemolytic streptococci were obtained 11 times; staphylococci 13 times; *B. bronchisepticus* once, and *B. lactis aerogenes* 3 times. *B. paratyphosus* B of human and of animal origin as well as *B. enteritidis* give identical cultural characteristics, and it is necessary to employ agglutination tests for their further identification into 3 separate subgroups. Bacilli belonging to the enteritidis paratyphoid B group previous to their more exact identification by agglutination are designated throughout this study by the letters PT and the number of the animal from which they were recovered. Organisms of the enteritidis paratyphoid B group were derived directly from the organs in every instance and not from the blood stream. The spleen was the most favorable site for the recovery of these organisms, as has been noted by Tenbroeck³ and others, while the streptococci and staphylococci were more frequently obtained from the heart blood. The members of the enteritidis animal paratyphoid B group were obtained by us only in those animals subjected to the more extreme variations in temperature. In these guinea-pigs, the infections were acute, and, as a rule, speedily terminated in death, but when the infectious process lasted for a longer period before death intervened, varied flora were obtained. The staphylococcus was obviously a postmortem invader.

In order to test whether streptococcus merely appeared in the terminal septicemia or whether any closer relationship of streptococcus and *B. bronchisepticus* to the paratyphoid B organisms could be

detected, 9 guinea-pigs, housed under favorable conditions, were injected intravenously in groups of 3 with initial doses containing approximately 4 billion of each of the 3 organisms in question. Each group was placed in a separate cage with a normal control animal.

The animals receiving single doses of *B. paratyphosus* B of animal origin and *B. bronchisepticus* died within 24 hours following the injection, and postmortem cultures from all 6 yielded only organisms giving cultural characteristics of the enteritidis paratyphoid B group. From the results obtained on injecting *Streptococcus equinus* into the 3 guinea-pigs of the 3rd group, streptococci appeared to be relatively innocuous, at least in comparison to the paratyphoid bacilli, as the animals survived 4 injections and postmortem cultures were negative. The control animals remained normal, and the anatomic findings in these paralleled the negative bacteriologic results. In addition to these results, it may be noted that we have not failed to recover the paratyphoid B bacillus from any of the guinea-pigs into which it had been injected, and that the animals invariably succumbed, although the dosage was considerably smaller than in the case of the streptococci. Whether in the group receiving *B. bronchisepticus* these organisms were overgrown in the test tube or were supplanted within the animal's body by the paratyphoid bacilli, could not be determined. These experiments apparently indicate that injection of sufficiently virulent bacteria other than those of the enteritidis paratyphoid B group may reduce the resistance of the animal enough to permit organisms of this group present in the intestinal tract to invade the blood stream and establish themselves in various organs of the host.

The organisms isolated belonging to the enteritidis paratyphoid B group were gram-negative, actively motile, rather short bacilli with rounded ends. They formed neither spores nor capsules. In plain broth, they produced a general turbidity with the exception of strain PT8 and PT40 in which the bacteria settled out as a heavy white sediment. On plain agar the organisms grew as flat, grayish, opalescent colonies, and little appreciable differences were observed in the character of these colonies. On Russell's agar slant the growth showed the typical acid butt and gas with alkaline slant. The acid butt turned alkaline at the end of a week. None of the strains formed indol in the Dunham's peptone solution up to the fourteenth day or liquefied gelatin at the end of 3 weeks, but all blackened lead acetate agar in 24 hours. The growth on potato was not characteristic. Litmus milk showed a slight primary acidity with a terminal alkalinity usually at the 5th day

and caseolysis or clearing up of the mediums at the end of 2 weeks. The organisms produced neither acid nor gas with the following test substances: lactose, saccharose, salicin, dextrin, inulin or glycerol. Fermentation tubes containing dextrose, levulose, arabinose, xylose, mannitol, dulcitol, or sorbitol produced acid and gas. In dulcitol and sorbitol broth, the fermentation was marked and rapid, and in many cases, the acidity of the mediums was gradually replaced by an alkalinity. This phenomenon was especially marked in the sorbitol broth. Inosite was not available in our laboratory at the time these organisms were cultivated, but Dr. Webster of the Rockefeller Institute to whom a culture of strain PT2 was sent, kindly tested its reaction and wrote that it fermented this substance. Six of the strains, namely PT40, PT8, PT3, PT2, PT1 and PT20, showing minor degrees of variation in growth, such as heavy sediment which settled out leaving a clear supernatant fluid in the broth in which the first 2 were grown, were respectively used to immunize 12 rabbits. The killed suspensions, although used in low concentrations, were highly toxic, and only 3 of the inoculated animals survived. The lethal dosage showed considerable variation, and in some of the animals initial doses containing 300,000 organisms were sufficient to kill rabbits weighing 2 kg. in 24 hours. With the immune serums derived from 3 strains—PT2, PT3 and PT40—agglutination tests were carried out with these strains of known bacteria: *B. enteritidis* (MT1) and *B. paratyphosus* (MT2), which were isolated from an epizootic of mouse typhoid, *B. pestis caviae*, *B. aertrycke* (mutton type), and *B. enteritidis* (Krumwiede);* *B. paratyphoid* B isolated from the lung of a hog, and *B. enteritidis*;** *B. suipestifer* and strain A5 of an animal *B. paratyphosus* B isolated from a guinea-pig epizootic;*** and 2 strains of human *B. paratyphosus* B designated Bodak Johnson and R.V.H. obtained from stock cultures of our bacteriologic department. All agglutination tests were made with formalized salt suspensions and were incubated for one hour in a thermostat at 56 C. After removal from the thermostat, the tubes were allowed to stand over night in an icebox, and the final results were read the following morning. Direct agglutination tests of the 3 strains failed to establish definitely the relationship of the 3 organisms, although subsequently agglutinin absorption experiments

* Received from Dr. Webster of the Rockefeller Institute. *B. pestis caviae* and *B. enteritidis* were originally isolated by Krumwiede, and *B. aertrycke* had been received from Schütze.

** Received from Professor E. O. Jordan of the University of Chicago.

*** Received from Dr. Katherine Howell of the Michael Reese Hospital of Chicago.

proved that PT40 was *B. enteritidis* and that PT2 and PT3 belonged to the animal paratyphoid group. Thus the failure of immune serums PT2 and PT3 to agglutinate in a dilution above 1:100, a suspension of PT40, as well as organisms of the enteritidis group with the single exception of MT1 (Rockefeller) indicates a difference which is not borne out by the results obtained when immune serum PT40 is used as the agglutinating agent. In the latter case, this relationship is only partially confirmed, since organisms of both the enteritidis and animal paratyphoid group are agglutinated, although the former is in a slightly higher titer. This double agglutination by immune serum PT40 may

TABLE 1
SIMPLE AGGLUTINATION WITH IMMUNE SERUMS PT2, PT3, AND PT40

Formalized Bacterial Suspension	Limit of Agglutination		
	Antiserum PT2	Antiserum PT3	Antiserum PT40
PT2.....	1,600	1,600	3,200
PT3.....	1,600	1,600	3,200
PT20.....	800	800	3,200
PT40.....	0	0	6,400+
<i>B. paratyphosus</i> B (hog lung) Jordan.....	800	1,600	3,200
<i>B. paratyphosus</i> B (mouse) MT2, from Rockefeller Institute.....	800	800	1,600
<i>B. paratyphosus</i> B (guinea-pig) A5, Howell and Schultz.....	800	1,600	3,200
<i>B. aertrycke</i> (Schütze) from Rockefeller Institute.....	800	1,600	3,200
<i>B. pestis caviae</i> (Krumwiede) from Rockefeller Institute.....	800	1,600	3,200
<i>B. enteritidis</i> (mouse) MT1, from Rockefeller Institute.....	800	400	6,400+
<i>B. enteritidis</i> (Jordan).....	0	0	6,400+
<i>B. enteritidis</i> (Krumwiede) from Rockefeller Institute.....	100	100	6,400+
<i>B. paratyphosus</i> B (human) strain B. Johnson.....	0	0	100
<i>B. paratyphosus</i> B (human) (R. V. H.).....	0	0	0
<i>B. bronchisepticus</i>	0	0	0

be due either to a natural immunity acquired against *B. paratyphosus* B occurring spontaneously in the rabbit or to group agglutinins occurring in conjunction with the specific agglutinin. The agglutinin absorption experiments recorded in table 2 leave no doubt as to the identity of PT2 and PT3 and of the fact that they belong to *B. paratyphosus* B of animal origin. It, furthermore, establishes the close relationship of the 2 strains isolated by us to the *B. paratyphosus* B recovered by Jordan from hog's lung, to the paratyphoid B bacillus, strain MT2, isolated by Webster, to *B. aertrycke* received from the Rockefeller Institute, and to the strain A5 *B. paratyphosus* B recovered by Howell and Schultz. Culture PT40 belongs to the enteritidis group. At present, no explanation can be offered for the irregular results shown by *B. enteritidis* MT1 with immune serums PT2 and PT3, although such irregularities in a simple agglutination test have been known to exist within this group.

Considerable difficulty, especially with *B. paratyphosus* B of animal origin, was experienced in producing immune serums of high titer in rabbits. This was partly due to the extreme toxicity of the killed and living organisms permitting only the use of small doses, and also the differences in antigenic properties possessed by various substrains. As shown by Arkwright⁹ and by Schütze,¹⁰ there is more or less correspondence between the agglutinating, absorbing and immune serum producing properties of such variants and the variations in their manner of growth in broth and on agar mediums. From the same original

TABLE 2
AGGLUTININ ABSORPTION TESTS WITH IMMUNE SERUMS PT2 AND PT40

Culture Tested	Titer Limit of Serum PT2 Saturated with					Titer Limit of Serum PT40 Saturated with	
	<i>B.</i> para- typhosus B, MT2	<i>B.</i> aertryeke (Schütze)	PT2	<i>B.</i> para- typhosus A5 Howell and Schultz	<i>B.</i> enterit- idis MT1	<i>B.</i> enterit- idis MT1	<i>B.</i> pestis caviae, Krum- wiede
PT2.....	0	0	0	0	800	0	0
PT20.....	0	0	0	0	800	0	0
<i>B.</i> paratyphosus B (guinea- pig), A5, Howell and Schultz.	0	0	0	0	800	0	0
<i>B.</i> aertryeke (Schütze) from Rockefeller Institute.....	0	0	0	0	800	0	0
<i>B.</i> pestis caviae (Krumwiede) from Rockefeller Institute....	0	0	0	0	800	0	0
<i>B.</i> suispestifer (Howell).....	0	100	0	0	0	0	0
<i>B.</i> paratyphosus B (mouse) MT2 from Rockefeller Insti- tute.....	0	0	0	0	0	0	0
<i>B.</i> enteritidis (mouse) MT1 from Rockefeller Institute....	0	0	0	0	200	0	800
<i>B.</i> enteritidis (Jordan).....	0	0	0	0	200	0	800

culture, colonies may be selected which give uniform turbidity in broth and smooth colonies on agar, while others form a heavy white sediment with a supernatant clear fluid in broth and a rough growth on agar. The strain PT40 isolated by us was characterized by the production of marked sedimentation in broth, and by agar colonies with slightly indented edges, and is, therefore, of the rough type. With only 2 doses each containing approximately 300,000 killed organisms of this strain, an immune serum causing agglutination in 1:6,400 dilution was obtained, although 10 intravenous injections of strains PT2 and PT3 (the last dose of which contained 7,000,000,000 killed organisms) produced immune serums giving agglutination in a dilution of 1:800 and

⁹ Jour. Path. & Bacteriol., 1921, 24, p. 36.

¹⁰ Jour. Hyg., 1921, 20, p. 330; Lancet, 1920, 198, p. 93.

1:1,600, respectively. Time did not permit extensive studies of the finer serologic characteristics of this rough variant, but, as has already been noted, direct agglutination tests, the results of which appear in table 1, gave indecisive differentiation between it and the paratyphoid B of animal origin. This finding agrees with the conclusion of Schütze¹⁰ that agglutination results with rough strains are not always to be trusted. It is, however, not impossible that immune bodies arising spontaneously from the presence of *B. paratyphosus* B may have been present previous to and during the immunizing process in antiserum PT40.

DISCUSSION

Although bacteria belonging to the enteritidis group have been isolated sporadically from infectious and pathologic conditions in various domestic and laboratory animals, it is only through the more extensive studies of Amoss,⁸ Webster,¹¹ O'Brien,⁵ Topley,² Howell and Schultz⁷ in the past few years that their importance in relation to the occurrence and spread of epizootics has been emphasized. While organisms of both the enteritidis and paratyphoid B types have been recovered from organs or heart blood of our animals, it has not been possible to recover either one or both of them from a high percentage of animals dying spontaneously. The occurrence of these bacilli in the intestinal tract of normal animals has been reported by several workers. Their presence in apparently normal guinea-pigs in our stock was evident from the fact that following a lowering of the resistance due to injections of pure cultures of *B. bronchisepticus* organisms of the enteritidis paratyphoid B group only were recovered in postmortem cultures. Many instances of recovery of organisms belonging to this group, subsequent to procedures tending to lower the animal's resistance, are recorded in the literature. On injection into 2 guinea-pigs of *B. mallei* and *B. typhosus*, respectively, MacConkey¹² obtained an organism indistinguishable from *B. enteritidis*. It is probable that the actively motile organism producing no coagulation in milk, which Klein¹³ recovered after injecting guinea-pigs with *Staphylococcus pyogenes-aureus* also belonged to the enteritidis paratyphoid group. Further evidence of the latency of these bacteria may be adduced from phenomena of like character which have been observed in domestic animals. Januscke,¹⁴ having recovered organisms belonging to the enteritidis paratyphoid B group from fowls after the

¹¹ Jour. Exper. Med., 1922, 36, p. 71.

¹² Jour. Hyg., 1905, 5, p. 333.

¹³ Twenty-Second Rep. Loc. Gov't Board, 1892-93, p. 267.

¹⁴ Centralb. f. Bakteriöl., I, O., 1922, 88, p. 518.

injection of tuberculin postulated that the presence of these in the blood stream and organs was due to a lowered resistance resulting from the tuberculin. Likewise Graham and Reynolds¹⁵ found that horses and mules were afflicted with "shippers disease," a pathologic condition in which *B. paratyphosus* B of animal origin was isolated, when the resistance of these animals was lowered by withholding food and water either experimentally or while the animals were in transit.

That streptococci occur frequently in the heart blood and organs at the time of death, is indicated by the high percentage of these organisms reported in postmortem cultures of guinea-pigs by Holman.⁴ The frequency with which Fredette¹⁶ and Richey and Goehring¹⁷ recovered streptococci in human agonal bacteremias demonstrates, however, that these organisms may occur in postmortem cultures as secondary invaders in the terminal septicemia.

The reasons underlying our failure to obtain positive cultures from a greater percentage of animals dying spontaneously are not clear. It is worthy of note that bacteria were recovered only from the acute cases which followed exposure to cold and rapidly terminated. When the fatal illness was prolonged, staphylococci and streptococci were frequently obtained, but rarely *B. paratyphosus* B. That the streptococcus used in our studies was relatively innocuous, at least in comparison to organisms giving the cultural characteristics of the enteritidis bacillus, was indicated by the fact that repeated doses equal in amount to a single fatal dose of *B. paratyphosus* B of animal origin produced no appreciable ill effects. It is, of course, possible that the streptococci may supplant the more virulent paratyphoid B bacillus under unknown conditions obtaining in the body immediately previous to death.

It seems evident that all of the guinea-pigs used in our laboratories were harboring in their intestinal tracts organisms of the animal paratyphoid enteritidis group and that these intestinal inhabitants needed only a slight lowering of the host's resistance to insure their spread throughout the body. Whatever the source of the original infection, it is obvious that if a single member of a group of stock rodents becomes infected, such an animal can not only reinfect itself through ingestion of fecally contaminated food, but may likewise spread the infection to other members of the group. With reinoculations of the intestinal tract, elimination of bacilli in the feces will occur either continuously or inter-

¹⁵ Jour. Am. Vet. Med. Assn., 1919-20, 56, pp. 378, 489 and 586.

¹⁶ Jour. Lab. & Clin. Med., 1916, 2, p. 180.

¹⁷ Jour. Med. Res., 1918, 33, p. 421.

mittently over long periods of time, simulating a "carrier state." Under normal conditions, these intestinal inhabitants are apparently relatively innocuous, but as the resistance of the animal is lowered through various causes, such, for example, as changes in temperature, bad housing and poor food, there is an invasion of the blood stream and organs. The influence which such fluctuations in environmental conditions exert on the animal's resistance suggests a basis for the subinfections postulated by Adami.¹⁸ The manner in which the defense mechanism of the intestinal mucosa is overcome in these mild bacterial invasions is not clear, although it is obvious that ulcers of the stomach, such as we observed, offer a ready portal of entrance for the large numbers of bacteria present in fatally terminating infections.

CONCLUSIONS

1. Organisms of the enteritidis paratyphoid B group have been isolated from organs of guinea-pigs of our stock dying spontaneously.
2. The organisms have been identified by agglutination tests as *B. enteritidis* and *B. paratyphosus* B of animal origin, and are similar to strains reported by several other workers on this continent.
3. Rodents once infected with these bacteria may continue over long periods of time to reinfect themselves through ingestion of fecally contaminated food, thus simulating a true carrier state.

II. PATHOLOGIC CHANGES IN VARIOUS ORGANS WITH SPECIAL REFERENCE TO HYDROPIIC DEGENERATIONS OF THE ISLETS OF LANGERHANS

Comparatively few publications have dealt with the pathologic changes produced in tissues and organs by the enteritidis paratyphoid B group. Among the histologic reports may be mentioned that of Pappenheimer and von Wedel,¹⁹ which contains a complete review of the previous literature and the recent article of Howell and Schultz.²⁰

The second part of our report deals with the pathologic changes found in the organs of the 47 guinea-pigs discussed. Fairly complete necropsies were made as soon after death as possible, so that any degenerative lesion which was common to several animals cannot be explained on the basis of postmortem change. Three fixatives were employed in order that special staining methods might be used. These

¹⁸ Jour. Am. Med. Assn., 1899, 2, p. 1572; Brit. Med. Jour., 1914, 1, p. 77.

¹⁹ Jour. Infect. Dis., 1914, 14, p. 180.

²⁰ Ibid., 1922, 30, p. 516.

were Zenker's fluid; Zenker's fluid in which 10% formalin was substituted for acetic acid and a solution containing 2.5% potassium bichromate and 10% formalin. Paraffin sections stained with hematoxylin and acid fuchsin were used for the routine microscopic examinations.

While the degree and intensity of the lesions varied considerably in different experiments, it has seemed best for purposes of brevity to present a digest of the pathologic changes found in the entire group of guinea-pigs, and then to separate the more characteristic lesions which occurred respectively in the kidney, liver, spleen, and pancreas.

DIGEST OF GROSS AND MICROSCOPIC PATHOLOGIC CHANGES

Thymus Gland.—Grossly, the thymus gland appeared normal. Microscopically, there occurred in some instances numerous large, round, swollen cells filled with fine granules or homogeneous material and more or less polymorphonuclear leukocytic infiltration.

Lungs.—The lungs appeared congested and anthracotic, and in some cases individual lobes resembled a state of red hepatization. The sections disclosed marked congestion and swelling of the alveolar walls, with many endothelial cells and occasional hemorrhages. Frequently, the epithelial lining of the small bronchioles was degenerated and disintegrated.

Heart.—Sections of heart from nine animals showed slight edema with congestion. In places, the fibers were swollen, sometimes filled with clear vacuoles, and the cross striations stained palely.

Stomach and Intestines.—The stomach appeared normal in the gross in all except 5 animals. In these, large ulcerations were located on the greater curvature near the pylorus. In microscopic preparations, the ulcerated areas extended down to the muscular coat and consisted chiefly of cellular debris interposed with shreds of fibrin and many degenerated neutrophils.

In several instances, the intestines were congested, and Peycr's patches could be readily distinguished. Parts of the small intestine were often translucent and filled with a yellowish fluid. Sections showed congestion, intense edema and degeneration of the epithelial lining. Lymphocytes, plasma cells and a few endothelial cells containing phagocytosed red blood cells were occasionally seen.

Liver.—The liver was usually slightly enlarged and congested. Frequently, irregular gray flaky-like areas varying from 1 mm. to 2 cm. in diameter were scattered over the surface and sometimes extended far into the parenchyma of the liver. Typical abscesses were found in several cases, the largest of which was 4 mm. in diameter.

Microscopically, the liver cells were swollen, granular and contained a considerable amount of brown pigment. Congestion was marked and the endothelial lining of the sinusoids was, as a rule, markedly swollen.

Apparently, the earliest typical lesions were areas of focal necrosis infiltrated with endothelial cells which were identical with the focal necrosis characteristic of typhoid infections in man. The margins of the necrotic areas were sometimes infiltrated with polymorphonuclear leukocytes, and all stages of progressive lesions of these types were seen up to definite abscess formation. Bile canaliculi were often prominent in the vicinity of the central veins and in

the areas surrounding the focal necroses. A few large areas of diffuse necrosis involving parts of two or more lobules were seen. Leukocytes were also scattered diffusely throughout the sinusoids.

Biliary cirrhosis varying in extent, occasionally reaching an extreme degree and giving rise to destruction of considerable hepatic tissue with resulting distortion of the liver architecture, was present. The bile ducts showed hyperplasia and frequently their lumina contained sero-albuminous fluid.

The gallbladder was normal in all animals examined except one, and this contained a fibrinopurulent exudate. The submucosa was edematous; the glandular epithelium showed degeneration and beginning desquamation and, in places, small abscesses.

Pancreas.—In none of the animals were any gross pathologic changes of the pancreas noted. Microscopically, the majority of the sections showed striking changes in the islets of Langerhans with correspondingly little alteration in the acinar elements. A complete description of the changes in the islets is included in the discussion of the pancreatic lesions and is omitted here to prevent repetition.

Spleen.—The most frequent pathologic change occurring in the animals studied was the enlarged congested purple spleen. In two instances, it was covered with a fibrinous exudate, and occasionally minute abscesses could be recognized.

Microscopically, the spleen was characterized by a marked hyperemia, a decrease in lymphoid tissue, and marked phagocytic activity of the numerous endothelial cells. The splenic nodules were represented by scattered patches of small lymphocytes. The sinusoids were distended with plasma and erythrocytes. Many large endothelial leukocytes filled with red cells and blood pigment were observed free in the blood channels as well as attached to walls of the sinuses. In several animals, neutrophils with a few eosinophils were observed diffusely distributed throughout the sinusoids, as well as massed in small necrotic areas. A few large typical abscesses undergoing encapsulation were encountered. The splenic pulp was frequently invaded by fibroblasts, and in a few instances scarring had progressed to a considerable extent.

Kidney.—Generally, in the gross the kidney remained normal in size, the capsule stripped readily, and little scarring or pitting could be recognized. In the more acute cases, petechial hemorrhages occurred beneath the capsule. On section, the surface showed cloudy swelling and the cortex appeared yellowish, frequently with hemorrhagic streaking.

Microscopically, the most prominent changes occurred in the glomeruli, convoluted tubules and the ascending limbs of the loops of Henle. The glomerular capillaries showed a marked congestion and frequently aneurysmal dilatations. Intraglomerular and extraglomerular hemorrhages were likewise frequent. In the inoculated animals, the glomerular vessels often contained embolic masses of bacteria with resultant thrombosis and necrosis of part or all of the involved glomerulus and the subtended tubules. These were infiltrated with lymphocytes, plasma cells and endothelial leukocytes and occasionally a few neutrophils. When the bacteria were less numerous, a hyperplasia of the endothelial lining of the glomerular capillaries of varying intensity occurred. Serous exudate and frequently also erythrocytes or necrotic debris were observed in the capsular space.

The parietal epithelium of Bowman's capsule showed lesions varying in intensity from a granular degeneration confined to the cells of the lower half (in proximity to the neck) to a complete degeneration and desquamation of the

entire lining. No proliferation of the epithelium of the capsule was encountered, and although early scar formation occurred within many of the glomeruli, complete replacement of this structure was not observed. Periglomerular envelopes of connective tissue were frequent. A marked degree of parenchymatous degeneration was noted in the proximal convoluted tubules and to a somewhat less degree in the distal convoluted tubules and ascending limb of the loop of Henle. The lumina of the tubules rarely contained definitely formed casts, but cellular debris was common. Scattered patches of scar tissue were found surrounding the interlobular and arcuate vessels, in and about the glomeruli, just beneath the capsule of the kidney and irregularly distributed throughout the medulla.

Suprarenals.—In gross appearance, the suprarenal gland varied from normal to a marked congestion. Microscopically, a generalized congestion with cellular degeneration was present and was most intense in the medulla. Swollen cells containing clear vacuoles varying in size and showing progressive stages of chromatolysis were prominent in the zona fasciculata and less marked in the zona reticularis. The zona glomerulosa exhibited the least degenerative changes, although the septums frequently seemed thickened. Occasionally the extreme congestion in the zona fasciculata had given place to hemorrhagic areas, with an infiltration of endothelial cells and fewer neutrophils.

Ovaries.—Microscopically, a few of the 10 animals studied showed pathologic changes in the Graafian follicles. The cells forming the membrana granulosa and the discus proligerosus were degenerated, and many of them were in the stage of dissolution, the resulting debris of which filled the centrum folliculi.

Testes.—The testes were enormously swollen and soft and pulpy. The sections from 9 cases showed extreme edema and degeneration, especially marked at the center of the seminiferous tubules decreasing toward the basal membrane. The efferent ductules and the epididymal tubules were filled with cellular detritus and desquamated cells from the seminiferous tubules, but contained no matured spermatozoa. The cells lining the epididymal tubules, efferent tubules and the tubuli recti were often pale and vacuolated.

DISCUSSION

It is obvious from the descriptions that the changes found agree in a general way with the lesions caused by *B. enteritidis* and *B. paratyphoid* B infections as already described by other investigators.

The renal lesions observed by us are similar to those reported by Pappenheimer and von Wedel¹⁹ and by Howell and Schultz.²⁰ Indeed, a comparison of the lesions described here with those produced by other workers by injection, not only of the same bacteria but diverse types as well, reveal certain similarities, the most outstanding of which are the acute degenerations and the lack of proliferative reactions in the capsule.

The highly toxic qualities of the animal paratyphoid *B. bacillus*, as demonstrated by injections of small doses, as for example, 500,000 bacteria per kilo animal, in comparison to the relatively large doses of such bacteria as streptococcus and *B. bronchisepticus*, raise the doubt as to whether the latter may be solely responsible for the lesions fre-

quently attributed to them, since it is practically impossible to rule out the presence of the enteritidis group in laboratory animals of the rodent type. The presence of these latent organisms was shown in 3 animals inoculated with *B. bronchisepticus* and in which *B. paratyphosus* B alone was recovered from the organs. Apparently, the injection of *B. bronchisepticus* served to lower the animal's resistance sufficiently to permit the latent *B. paratyphosus* B strains not only to invade the blood stream and establish themselves within the various organs, but also to outgrow the injected bacteria. Similarly, exposure to lowered temperatures invariably resulted in widespread foci of inflammation due to the dissemination of these organisms throughout the body of the host. Because of the prevalence of such intestinal infections among animals, as evidenced by bacteriologic findings in numerous epizootics, we believe it is not possible to conclude definitely without concomitant bacteriologic control studies that exposure to cold, such as reported by Seidel,²¹ or abnormal diets as suggested by Newburgh,²² are sufficient per se to produce acute nephritis or nephrosis.

Pappenheimer and von Wedel¹⁹ considered areas of focal necrosis in the liver to be characteristic of paratyphoid lesions in laboratory animals, and Howell and Schultz²⁰ noted the frequent occurrence of liver abscesses in animals dying in an epizootic reported by them. The abscesses undoubtedly have their origin in the areas of focal necrosis. It is interesting in this connection that Libman,²³ in his report of a human infection with a similar organism, described areas of focal necrosis with an infiltration of polymorphonuclear leukocytes. Too much stress is not laid on the occurrence of biliary cirrhosis, since several instances of coccidial infection were found in the group of animals.

The intense phagocytic activity in the spleen under the stimulus of this organism is confirmatory of the necropsy reports of Trautman,²⁴ Pappenheimer and von Wedel,¹⁹ and Howell and Schultz,²⁰ although the occurrence of abscesses was less frequent in our animals than in those studied by the last mentioned authors.

The lesions noted in the pancreas, and especially in the islets of Langerhans, were so remarkable that it was deemed advisable to discuss them somewhat in detail.

²¹ Deutsch. med. Wchnschr., 1908, 34, p. 441.

²² Arch. Int. Med., 1919, 24, p. 359.

²³ Jour. Med. Res., 1902, 8, p. 168.

²⁴ Ztschr. f. Hyg. u. Infektionskr., 1906, 54, p. 104.

The literature dealing with the enteritidis paratyphoid B group of infections reveals little of interest regarding morphologic changes in the pancreas. Although a wide range of animals, including guinea-pigs, rabbits, mice, rats, chickens, calves, horses and mules, have been studied, the necropsy reports either do not include the pancreas or record it as normal. O'Brien²⁵ states that in an epizootic of guinea-pigs from which *B. aertrycke* was isolated, the pancreas was congested in a few animals, and Pappenheimer and von Wedel¹⁹ found that in a typhoid-like epidemic caused by an organism similar in cultural characteristics to *B. enteritidis* and *B. typhi-murium* A, the pancreas was normal. In a human case due to an organism identified as *B. paracolon* (later classed with the enteritidis paratyphoid B group), Libman²³ gave the following necropsy findings: "Pancreas, slight grade of pancreatitis; islands of Langerhans not involved; intima of arteries thickened."

As the pancreas was removed only in the later part of our series, the present study was limited to 36 guinea-pigs. Five of these were normal controls, 3 had been injected with small doses of the animal paratyphoid B bacillus, 1 with *B. bronchisepticus*, a 3rd received an aqueous extract of nonhemolytic streptococcus containing a small proportion of disintegrated bacteria, and the remainder died spontaneously.

All of the important lesions of the pancreas appeared to be located in the islets of Langerhans, and were microscopic in character. Under low power magnification, the altered islets, because of their pale color and transparency, appeared in marked contrast to the surrounding glandular tissue. The lack of staining was due to a swelling and vacuolation of the individual cells, and reached a maximum in those islets in which the degeneration was at its height and where many of the cells had disintegrated. In a pancreas in which the process was well developed, islets consisting of even one or two cells could be readily distinguished at a glance. On higher magnification, the degenerated cells were found to be of two types—one hydropic, the other very finely granular. In the hydropic degeneration, the cells were swollen to several times their original size; the cytoplasm consisted of a fine granular network enclosing in its meshes clear vacuoles of varying sizes, and the nucleus was generally large with palely staining chromatin although occasionally it appeared smaller than normal and stained a deep blue. Frequently, the thread work had become almost invisible, and the vacuoles had

²⁵ Jour. Hyg., 1910, 10, p. 231.

reached an enormous size. This stage always preceded disruption of the cell membrane, which resulted in a karyolytic nucleus surrounded by a small mass of somewhat granular material having a frayed irregular margin. Finally, these remnants became reduced to spaces partially filled with cellular detritus in which no formed structure could be recognized. In extreme cases, 50% of the cells had undergone disintegration. In the second type of degeneration, the cells remained about normal in size, but contained a shrunken pyknotic nucleus staining deeply with hematoxylin, while the cytoplasm stained a fairly uniform deep pink. The proportion of these two types of degenerated cells in an islet varied in different animals. In a few, practically all of the islet tissue was composed of pale hydropic cells, but in the majority of our guinea-pigs the hydropic cells were grouped about the periphery of the islets. It was not possible to determine definitely whether these degenerated cells were of the alpha or beta type, since in our hands the specific technic and stains recommended by Bensley²⁵ and Lane²⁶ did not give clear cut results in differentiating the granules. This had likewise been the experience of Martin²⁷ and Homans²⁸ in the study of pathologic pancreatic tissue. No hemorrhages were observed in the islets and with careful search with the oil immersion lens, no mitotic figures were found.

The condition of the acinar cells varied greatly. In more than half of the animals, these were normal. Fatty degeneration in varying degree was observed in a few animals. In some, this fatty change in the cells of the acini was combined with a condition of apparent interstitial edema which was recognized by a retraction of the parenchymatous tissue from the reticular supporting network. This process, similar to that designed by Oertel²⁹ as an essential necrosis and probably in large part due to a shrinking of the parenchymatous cells, may give rise to a series of spaces around the glandular tissue measuring from 25 to 40 microns.

No fibrotic change or hyaline degeneration such as reported by Opie³⁰ in the human islets was observed by us. This may be explained by the acuteness of the lesion in our series of animals.

Although pathologic changes in the islet cells of the nature of a hydropic degeneration had previously been noted in the pancreatic

²⁵ Am. Jour. Anat., 1911-12, 12, p. 297.

²⁶ Ibid., 1907-08, 7, p. 409.

²⁷ Jour. Metabol. Res., 1922, 1, p. 43.

²⁸ Jour. Med. Res., 1914, 30, p. 49.

²⁹ Ibid., 1919, 40, p. 289.

³⁰ Diseases of the Pancreas, 1903.

islets of patients suffering from diabetes, Weichselbaum³¹ was the first to report a series of cases in which hyperglycemia was correlated with hydropic degeneration of the islets. This author's observations have since been confirmed by the experimental work of Homans²⁸ and of Allen³² and his co-workers. The latter investigators believed that these morphologic changes resulted from depletion of the beta cells through overstimulation, since a partial pancreatectomy with subsequent feeding of high carbohydrate diet called forth specific hydropic changes in the islets. The lesions observed by us are identical with those produced by Allen, although the explanation of the method of production is not so simple in our case. That the islet lesions found in guinea-pigs dying spontaneously and from which organisms of the enteritidis paratyphoid group have been isolated or which have been inoculated with similar bacteria are due to these bacteria or their products, now seems undoubted. The immediate inciting cause is toxic rather than inflammatory in character, as no cellular exudate can be demonstrated in the islets. Neither the significance nor the exact mechanism of production of the two types of cellular degeneration are clear. The process leading to the terminal stage in either case seems to be distinct. The pathologic changes are instituted in the islet tissue long before any degenerative change of the acinar tissue can be detected. While the earlier and more intense destruction of the islets may be partially explained by the great vascularity of the islet tissue enabling toxic substances to come into intimate contact with these cells, intrinsic affinities toward specific toxic substances must likewise be a factor since few other bacteria or toxins apparently exert a similar action. It may be mentioned that the work of Dr. Maud L. Menten, which has been in progress for some months in these laboratories, demonstrates beyond question that these lesions are correlated with variations in blood sugar.

CONCLUSIONS

The liver, spleen, kidneys and pancreas of guinea-pigs dying spontaneously, and from which organisms of the enteritidis paratyphoid B group were recovered, showed characteristic pathologic changes. The most marked lesions occurred in the pancreas, in which the islets of Langerhans showed hydropic degeneration of marked degree.

³¹ Wien. klin. Wchnschr., 1911, 24, p. 153.

³² Jour. Metabol. Res., 1922, 1, pp. 5, 75.

ANAEROBIC SPORE-BEARING BACTERIA OF THE HUMAN INTESTINE IN HEALTH AND IN CERTAIN DISEASES

MORTON CHARLES KAHN

From the Department of Hygiene, Cornell University Medical College, New York City

The group of diseases here considered are among those which clinicians have long surmised may be due to, or in some way definitely correlated with, changes in the intestinal flora brought about by one or another species of bacteria. This is particularly true of the morbid conditions from so-called intestinal toxemia, chronic diarrhea, certain skin eruptions of otherwise unknown etiology, the more obscure joint affections, and to some extent of pernicious anemia.

Nearly all of these diseases have been studied extensively from a dietetic point of view without any definite results as to their causation. They have also been investigated on the assumption that they are primarily infectious in the true sense of the word. At the suggestion of Dr. J. C. Torrey, I have examined the intestinal flora of the series of cases here presented, for the presence of anaerobic spore-bearing bacteria to determine whether any correlation could be demonstrated between the types and numbers of these organisms and the clinical manifestations.

The purposes of this study then are as follows:

1. With the improved technic and added information at our command, to present a thorough bacteriologic analysis of the anaerobic spore-bearing bacteria as found in the feces of adult persons diagnosed as having one of the following conditions: (a) "intestinal toxemia," (b) chronic diarrhea, (c) chronic arthritis of unknown etiology, (d) chronic eczema and urticaria, and (e) pernicious anemia.

2. To present a bacteriologic analysis of the anaerobic spore-bearing bacteria found in the feces of normal persons.

3. To correlate, if possible, certain of the untoward symptoms presented in the pathologic cases with the types and numbers of anaerobic spore-bearing bacteria isolated.

4. To present a technic suitable for isolating, purifying and cultivating anaerobic spore-bearing bacteria from intestinal sources.

5. To present the more important differential methods now used in classifying and grouping anaerobic spore-bearing bacteria.

HISTORICAL

INTESTINAL TOXEMIA

Early conjectures concerning the correlation of this group of bacteria with symptoms supposedly arising from intestines heavily infected with them followed two definite and contemporary attitudes. The first concerned itself chiefly with putrefying processes within the intestinal tube thought to be brought about by the strongly proteolytic anaerobes, known up to recently more or less interchangeably as *B. oedematis maligni*, *B. sporogenes* and *B. putrificus*. These organisms rapidly digested protein, and it was thought that incomplete digestion of nitrogenous substances and stasis taking place within the intestine offered an especially favorable medium for their development and activity. The bacteria were suspected of exerting their harmful action not by any outstanding pathogenic or invasive ability, but by secondary features incidental to their protein metabolism. It was felt that the by-products of such protein digestion by bacteria, namely, indole, skatole, phenole, mereaptan and hydrogen sulphide, were absorbed, and if such a condition were allowed to continue, grave symptomatic manifestations would become apparent and morbid conditions result.

The second speculation had in the main to do with the saccharobutyric type of fermentation typified by the presence and sometime overgrowth of *B. welchii* and closely allied species. The irritating action of butyric acid produced in relatively large amounts by *B. welchii*, as well as a definite and potent poison which bacteria of this species are able to elaborate in the presence of certain types of culture medium, accounted for the morbid processes associated with these intestinal bacterial conditions to the satisfaction of some of the earlier investigators.

Among the first to call attention to this problem of supposed intestinal toxemia was Senator¹ who, as early as 1868, claimed that decomposition of protein within the alimentary canal resulted in the formation of substances highly toxic to the host. Bouehard² also held to this theory, and pointed out that bacteria within the intestine may have been responsible for the breaking down of the undigested proteins, the toxic products of which were subsequently absorbed. Bouehard claimed also that the amount of putrefactive by-products eliminated in the urine was a measure of the degree of putrefaction in the intestine. Bienstock³ called attention to certain highly putrefactive types of bacteria which he isolated from human feces, and this gave further credence to the theories of Senator and Bouehard. Hammer⁴ found that anaerobic putrefying bacteria were more common in the stools of persons fed on a mixed diet containing meat than in those from persons subsisting on vegetables alone. Bienstock⁵ claimed from his study of the feces of several normal persons, that *B. putrificus* was never present in the intestines of normal persons, and its presence in a specimen attested to the dangerous type of flora in the subject harboring it. Tissier and Martelly⁶ reported finding and isolating an organism similar to Bienstock's *B. putrificus* from the meconium and therefore were not prone to lay much stress on Bienstock's theory. Salus⁷ found spores of

¹ Berl. klin. Wchnschr., 1868, 5, p. 264.

² Compt. rend. Soc. de biol., 1884, 1, p. 665.

³ Ztschr. f. klin. Med., 1884, 8, p. 1.

⁴ Ztschr. f. Biol., 1897, 35, p. 355.

⁵ Arch. f. Hyg., 1901, 39, p. 390.

⁶ Ann. de l'Inst. Pasteur, 1902, 16, p. 865.

⁷ Arch. f. Hyg., 1904, 51, p. 97.

putrefying anaerobes in stools from normal persons, but in small numbers only. Passini⁸ detected *B. putrificus* in the meconium of normal babies on several occasions, later finding this anaerobe more common as a rule in the stools of bottle-fed than of breast-fed babies. He did not at that time associate *B. putrificus* with digestive disturbances found in infants. In a more recent publication, Bienstock⁹ was again unable to find *B. putrificus* in the feces of normal persons, and this led him to reassociate it with pathologic processes supposedly starting from the metabolic activities of this and allied types of anaerobes in the intestine.

Perhaps the most definite views in regard to the correlation of proteolytic anaerobes and derangements from an intestinal source were taken by Rettger¹⁰ and Herter.¹¹ Rettger claimed that putrefaction was entirely the work of the anaerobes and that no other type of bacteria was concerned. The best known of the proteolytic types were then, according to Rettger, the bacillus of malignant edema, *B. putrificus*, and the bacillus of symptomatic anthrax. He said, further, that except in a few rare instances, putrefactive organisms of the foregoing types had not been observed in the feces of normal persons. Rettger was then also of the opinion that *B. enteritidis-sporogenes* of Klein was regularly present in human feces, and although commonly regarded as being a fermentative type of anaerobe, he considered it probable that it may have been partly responsible for putrefactive changes in the intestine. Rettger was inclined also to think that *B. coli* and *B. lactis-aerogenes* were not harmful to the intestine. On the contrary, he assumed that their presence in a putrefying medium was a hindrance to the action of the putrefactive anaerobes. *B. oedematis-maligni* here referred to by Rettger is now taken to be *B. sporogenes*, and Klein's *B. enteritidis-sporogenes* is recognized as *B. welchii* by most present-day workers.

Herter considered *B. putrificus* and *B. oedematis-maligni* inhabitants of only strongly putrefactive intestines. He was able to isolate both forms abundantly from the feces of persons suffering from certain types of acute and chronic intestinal derangements of an obscure etiology, but only seldom, and then sparsely, from the stools of normal persons. Herter claimed further that in every instance in which *B. putrificus* was present in bouillon flasks which were prepared by growing the mixed fecal flora from cases of intestinal putrefaction, there was found also methyl mercaptan. This observation corresponded to another made by him that *B. putrificus* in peptone bouillon is capable of making mercaptan. On the other hand, Herter stated that he was not always able to isolate *B. putrificus* from cases in which the methyl mercaptan reaction was obtained, and for this reason he believed that organisms other than *B. putrificus* and kindred anaerobes may have been concerned in producing the pathologic conditions reported.

The powerful proteolytic properties attributed to *B. putrificus* by Herter (he considered it the most energetic anaerobe known in respect to its ability to digest protein) leads us to believe that his cultures were highly contaminated with *B. sporogenes*.

Rettger,¹² after examining about 50 normal stools for the presence of anaerobic spore-bearers, saw fit to modify the views published by him in 1906. In this second investigation, he found *B. putrificus* present in 20% of the

⁸ Ztschr. f. Hyg. u. Infektionskrank., 1905, 49, p. 135.

⁹ Ann. de l'Inst. Pasteur, 1906, 20, p. 497.

¹⁰ Jour. Biol. Chem., 2, 71, p. 1906.

¹¹ Bacterial Infections of the Digestive Tract, 1907.

¹² Jour. Biol. Chem., 1908, 4, p. 45.

subjects studied, the bacillus of malignant edema, probably *B. sporogenes*, present in 7.4% and *B. enteritidis sporogenes*, probably *B. welchii*, present in 32% of the 50 cases examined. These findings led Rettger to conclusions more in harmony with present-day views that stools from normal persons may contain types of putrefactive anaerobes. He, however, was able to isolate them in small numbers only, and concluded that these anaerobes existed in the intestines as spores, and in this form resisted the unfavorable environment of the human intestine. In certain kinds of digestive disturbances, Rettger thought it highly probable that the anaerobes took advantage of the new conditions afforded and developed to such an extent as to cause excessive putrefaction. He was of the opinion that the normal human intestine exerted a marked suppressing action on the development of the anaerobic spore-bearer which might gain access to it.

Metchnikoff¹³ held more radical views on the relation of bacteria in the intestinal tract to human life, well-being and pathology than did any of the earlier workers in this field. His advocacy of the use of cultures of lactic acid-producing bacteria to combat what he considered untoward types of bacteria in the digestive tube is practically of household familiarity. In a more popular summary of his ideas published in the "Prolongation of Life" (1908), he definitely correlated premature senility and senility with the absorption from the intestine of toxic products elaborated by certain types of bacteria. The anaerobes concerned in this process were specified by Metchnikoff to be *B. putrificus*, *B. sporogenes* and *B. welchii*. Each of these species, he stated, actively formed indole, skatole and phenole, which, absorbed from the intestine, produced grave lesions in important organs such as arteries, liver and kidney.

Distaso¹⁴ claimed that constipation was due largely to the effect of toxins formed and absorbed from the large intestine as a result of the growth of bacteria. Among the organisms mentioned by him as having a part in this process are *B. sporogenes* and *B. welchii*, the latter being classed by him as an indole former. Friedman¹⁵ reported that intestinal stasis was always accompanied by an appreciable increase of *B. welchii* in the sporulating form. Tissier¹⁶ was of the opinion that the stools of persons who harbored large numbers of *B. welchii* became exceptionally compact when the carbohydrate of the diet was reduced to the minimum and protein greatly increased.

The literature concerning the relation of *B. welchii* to intestinal putrefaction is by no means definite. This is due to the confusion and differences of opinion as to whether or not *B. welchii* is able to split protein. Among the workers prone to classify *B. welchii* as a putrefactive species are Herter,¹⁷ Metchnikoff,¹³ Louis Melikov,¹⁷ Tissier,¹⁶ Rettger,¹⁰ and Lotti.¹⁸ Shattenfroh and Grassberger¹⁹ are more in accord with present-day investigators who now, almost universally, consider *B. welchii* primarily a carbohydrate-splitting type with exceedingly weak, if any, protein-cleaving ability.

In accord with Bouchard's earlier theory, Herter stated that indicanuria was evidence of intestinal putrefaction. He claimed that no difficulty was encountered

¹³ Compt. rend. Acad. d. sc., 1908, 147, p. 576; Ann. de l'Inst. Pasteur, 1908, 22, p. 929; 1910, 24, p. 755; 1913, 27, p. 893.

¹⁴ Centralbl. f. Bakteriöl., 1912, 62, p. 433.

¹⁵ Tr. Chicago Path. Soc., 1901, 5, p. 172.

¹⁶ Ann. de l'Inst. Pasteur, 1912, 26, p. 522.

¹⁷ Compt. rend. Soc. de biol., 1913, 76, p. 229.

¹⁸ Ann. d'ig. sper., 1919, 19, p. 75.

¹⁹ München. med. Wchnschr., 1900, 47, p. 1032.

in producing this condition when large quantities of meat were fed to dogs. In such cases, he found throughout the large intestine and lower ileum moderate or large numbers of anaerobic spore-bearing, butyric acid-producing bacteria, as well as colon bacilli. He stated that *B. welchii* composed the large majority of these anaerobic organisms and that the indicanuria was produced largely as a result of their activity. Herter thus explains the headaches, flatulence and increase of indican in the urine following the imperfect action of a cathartic in humans. The action of the cathartic causes proteins and peptones to be swept from the small to the large intestine where the former is attacked by anaerobic bacteria, if they be present. In persons who harbor large numbers of *B. putrificus* or *B. welchii*, such an occurrence would be likely, according to Herter, to result in the appearance of relatively large volumes of indican in the urine.

Herter¹¹ was also of the opinion that *B. welchii* was largely responsible for a specific type of intestinal disturbance which he designated "saccharo-butyric fermentation." "This form of intestinal derangement is characterized by a chronic putrefactive process having its seat primarily in the large intestine and lower ileum, and is due to the action of a large number of strictly anaerobic bacteria, butyric acid-producing, capable of multiplying by spore formation. The organism most commonly concerned in a large majority of these cases is *B. welchii*, although *B. putrificus* is often found. *B. welchii* is, however, usually the dominant form and often the exclusive anaerobe present." Such stools, Herter says, are soft, light in color, due to the reduction of bilirubin, low in specific gravity, due to gas formation, and have the odor of butyric acid. The softness of the movement, he thought, was due in part to ammonium butyrate, which, he claimed, was formed in considerable amounts, and actively irritated the intestine. Herter claims to have found this abnormal condition widely spread among older children and adults.

ANAEROBIC SPORE-BEARING BACTERIA AND DIARRHEA

Klein²⁰ isolated an organism which he termed *B. enteritidis sporogenes* (now taken to be *B. welchii*) from the stools of patients affected in two epidemics of mild diarrhea which occurred in London. In the first epidemic, there were 59 cases and in the second 144. Andrews²¹ found this type of bacteria in the stools of patients from another epidemic of the same kind, embracing 44 cases, and also in 20 unselected cases of diarrhea. Hewlitt²² succeeded in isolating *B. enteritidis-sporogenes* from the feces of 12 patients with ulcerative colitis and from one patient with, what he termed, ordinary diarrhea. Shattenfroh and Grassberger²³ entirely discredited the conclusions of Klein and Andrews and were of the opinion that *B. enteritidis sporogenes* had nothing to do with the outbreaks of diarrhea from which the bacteria were isolated. Shattenfroh and Grassberger assumed this because Klein found the same type of organism in 9 of 13 samples of milk examined by him, and although Klein believed that the infections in the outbreaks were milk-borne, no infection resulted from the use of milk from the same source as the 9 samples in which he found the bacillus. According to Andrews,²⁴ *B. welchii* has usually been limited to the milder forms of diarrhea, being absent, as a rule, where the stools are of a choleric nature.

²⁰ Centralbl. f. Bakteriöl., 1895, 18, p. 737.

²¹ Lancet, 1899, 1, p. 8.

²² Tr. Jenner Inst. Prev. Med., 1899, 2, p. 70.

²³ Arch. f. Hyg., 1900, 37, p. 54.

²⁴ Annual Report of the Medical Officer of the Local Government Board, London, 1896-97, 26, p. 255.

Hewlitt²² found *B. enteritidis sporogenes* in the feces of 11 of 13 healthy persons, but concluded that it is quite conceivable that in outbreaks of diarrhea, the virulence of the bacteria as well as the number of them present would be greater than under normal conditions. He did not find evidence to hold this bacillus as the primary factor in epidemics reported by others. Glynn²⁵ found the spores of *B. enteritidis sporogenes* in the feces of healthy persons, and made the valuable observation that there was no difference in the virulence of these strains and those isolated from cases of diarrhea. Herter²¹ was strongly of the opinion that *B. welchii* is a causative agent of diarrhea. Savage²⁶ states that British authorities now consider Klein's bacillus as not having had anything to do with the outbreaks from which it was originally isolated.

Kendall and Day²⁷ held two factors essential for the proliferation of *B. welchii* in the intestinal tract: (1) an excess of carbohydrate which may be utilized by the gas bacillus; (2) a deficiency of bacteria capable of forming lactic acid from carbohydrates in sufficient volume to inhibit the growth of the Welch bacillus. When such a condition exists in the intestine, these observers claimed that the gas bacillus, which exerts its irritating action through the production of butyric acid, might cause diarrhea even to the extent of the presence of mucus, blood and pus. They found large numbers of *B. welchii* in diarrheal stools. For their numerical estimation, they depended on a small loopful of feces being sufficient to cause a positive milk reaction. This indicated an excess of *B. welchii* in the gastro-intestinal tract. Kendall and Day also claim that the Welch bacillus is sensitive to lactic acid, and, as indicated in the foregoing, bacteria capable of forming this type of acid from suitable carbohydrate tend to minimize the growth of *B. welchii*. This is contrary to our experience. We have found that when *B. acidophilus* was planted in milk medium and allowed to incubate for 18 hours and *B. welchii* then inoculated into the same tube, the characteristic stormy fermentation of *B. welchii* invariably occurred. This seems to indicate that *B. welchii* is not very sensitive to lactic acid, at least not to that produced by *B. acidophilus*, which is an aciduric type found in many normal intestines.

McCampbell²⁸ obtained a toxic and irritating effect from a solution of butyric acid equal in strength to that produced by a 24-hour milk culture of *B. welchii*. The importance of this observation was minimized by some observers who claimed that alkalinization of the butyric acid thus produced did not lessen the toxicity. They did not take into consideration, however, that sodium butyrate is also an irritating substance.

Among later investigators, Tenbroeck²⁹ is of the opinion that *B. welchii* plays only a subordinate rôle in summer diarrhea of children. Morris, Porter and Meyer³⁰ agree with this view, but state that *B. welchii* may be responsible for some of the grave symptoms of intoxication in infantile dysentery. Hines³¹ reports that the aerobic intestinal flora in 8 cases of diarrhea associated with intestinal lesions was definitely proteolytic, while in 2 cases of what he terms "fermentative diarrhea," the stools having a marked butyric acid odor, the aerobic flora was aciduric. In another similar case he reports proteolytic

²⁵ Thompson Yates Laboratory Reports, 1900-01, 3, p. 131.

²⁶ Milk and the Public Health, 1912.

²⁷ Boston Med. & Surg. Jour., 1912, 149, p. 753.

²⁸ Jour. Infect. Dis., 1909, 6, p. 537.

²⁹ Boston Med. & Surg. Jour., 1916, 174, p. 785.

³⁰ Jour. Infect. Dis., 1919, 25, p. 349.

³¹ Ibid., 1923, 32, p. 281.

potentialities for the aerobic flora. Hines found that spores of *B. welchii* were present in great numbers in stools having a proteolytic aerobic flora but absent in those with an aciduric flora.

The most outstanding treatise on the relation of *B. welchii* to diarrhea and other conditions is the careful and meritorious work of Simonds.³² After carefully weighing the evidence involved, Simonds presents the following summary:

Arguments in favor of *B. welchii* being considered as a causative agent in diarrhea: (1) Large numbers of *B. welchii* spores have been found in the stools in diarrheal cases; however the methods of the determinations have been crude; (2) persons so affected have been made worse by feeding carbohydrates, while improvement has usually followed a high protein diet.

Arguments against *B. welchii* being considered as a causative agent in diarrhea: (1) *B. welchii* is present in relatively large numbers in the feces of normal persons. This, however, may be accounted for by differences in individual susceptibility to infection with *B. welchii* and the virulence of the strain encountered. (2) *B. welchii* is present in abundance in meconium when the intestine of the infant is entirely without defense. Simonds considers that the promptness with which the aciduric flora is formed saves the child from disaster. (3) In children given cow's milk from birth, the meconial flora shows evidence of *B. welchii* for 15 to 30 days, but during this period *B. bifidus* and other aciduric organisms are gradually getting the upper hand. If the bottle-fed babies are given mother's milk for the first 8 days of life, *B. welchii* is not afterward found under normal conditions. (4) Numerous attempts to induce diarrhea by feeding cultures of *B. welchii* to laboratory animals, including some of the higher apes, has met with little success.

The preceding citations from the literature indicate that opinion is divided on this point, especially as concerns the relation of *B. welchii* to diarrhea in adults. No other anaerobe, as far as we know, has been associated with diarrhea.

ANAEROBIC SPORE-BEARING BACTERIA AND ARTHRITIS

Achalme³³ claimed to have isolated a bacillus, which is now taken to be *B. welchii*, from the heart blood and joints of a patient dead of acute articular rheumatism. Triboulet and Coton³⁴ reported isolating an organism of the same type from a patient fatally attacked with rheumatic endocarditis. Thiroloix³⁵ published an account of 5 cases of acute articular rheumatism in which he claims to have isolated the Achalme bacillus from the blood and from joints during life, and he claimed further to have been able to produce cardiac and joint lesions by injecting cultures of this organism. Savchenko³⁶ studied 6 cases of this same malady, and obtained from 5 of them *B. welchii* in pure culture. From the other he also isolated it, but associated with streptococci. Pic and Lesieur³⁷ found this bacillus in blood cultures taken from a case of acute articular rheumatism on 2 occasions. Hewlitt³⁸ succeeded in isolating the same type of organism from a rheumatic knee joint. Rosenthal³⁹ definitely

³² Monograph Rockefeller Inst., 1915, 5.

³³ Compt. rend. Soc. de biol., 1891, 43, p. 651.

³⁴ Compt. rend. Soc. de biol., 1897, 49, p. 1000; Le Rheumatisme Articulaire, Aigu en Bacteriologie, 1900.

³⁵ Compt. rend. Soc. de biol., 1897, 49, pp. 268, 945.

³⁶ Abst. klin. med. u. Bakteriolog., 1898, 5, pp. 558, 614.

³⁷ Jour. de Physiol. and de Path. General, 1899, 1, p. 1007.

³⁸ Lancet, 1901, 1, p. 705.

³⁹ Compt. rend. Soc. de biol., 1906, 58, p. 828; 1913, 74, p. 1104.

states that one strain of *B. welchii*, which he named "Anhamobacillus," is the causative agent of acute articular rheumatism. Aehalmé⁴⁰ reasserted the views taken by him at an earlier date to the effect that the organism isolated and described by him in 1894 is the cause of this disease. Rosenow⁴¹ obtained *B. welchii* in a number of cases from enlarged lymph glands draining joints showing lesions of arthritis deformans. Bose and Carriew⁴² have found spores of *B. welchii* on the skin of persons on various parts of the body, and believe that the reported findings of this organism in the blood and joints have been due to skin contamination. On the basis of this finding they are prone to discredit the theories concerning the relation of *B. welchii* to acute articular rheumatism and arthritis in general, as advanced by other observers. Simonds³² is inclined to support the opinions of Bose and Carriew.

PERNICIOUS ANEMIA

Herter⁴³ investigated the anaerobic spore-bearing flora of the stools of 17 patients with pernicious anemia, and found *B. welchii* to be the outstanding organism of this group, and much more numerous than in normal stools. Blair, quoted from Herter, stated that carnivora in the New York Zoological Garden frequently suffered from anemia of the pernicious type. Herter found *B. welchii* much more numerous in the feces of carnivora than in those of herbivora. The latter never have been known to suffer from anemia. Rettger¹⁰ noted a lack of putrefactive bacteria in stools from pernicious anemia cases. Klotz and Hollman⁴⁴ and Leroy⁴⁵ have pointed out the marked anemia and evidence of destruction of erythrocytes in patients with gas gangrene due to infection with *B. welchii*. Sehum⁴⁶ found that the serum of persons with *B. welchii* bacteremia gave positive spectroscopic tests for hemoglobin. Simonds³² studied the feces from 4 cases of pernicious anemia and found *B. welchii* far more numerous than in any other stools examined. Coates⁴⁷ theorizes on the relation of the intestinal flora to pernicious anemia as follows: (1) Delayed protein digestion allows an undue proportion of food residue to remain in the lower part of the ileum in a form readily prone to bacterial decomposition, thus providing a material likely to form toxins which may be hemolytic. (2) Neurotoxins may be formed, causing subacute, combined degeneration of the spinal cord. (3) Toxins may be formed stimulating the bone marrow to produce abnormal types of erythrocytes.

Coates found ulceration of the intestine in quite a number of cases of pernicious anemia, and points out that toxins are more permeable to an abraded than to a normal surface. Unfortunately, he made no culture from these ulcers.

ANAEROBIC SPORE-BEARING BACTERIA IN NORMAL STOOLS

Tissier and Martelly⁶ isolated an organism similar to Bienstock's *B. putrificus* from the stools of normal persons. Salus⁷ detected putrefying anaerobes (now taken to be *B. sporogenes*) from a similar source. Herter¹¹ and Rettger¹² isolated proteolytic anaerobes from the feces of normal persons

⁴⁰ Compt. rend. Soc. de biol., 1913, 75, p. 82.

⁴¹ Tr. Chicago Path. Soc., 1914, 9, p. 115.

⁴² Compt. rend. Soc. de biol., 1913, 74, p. 1229.

⁴³ Jour. Biol. Chem., 1906-07, 2, p. 1.

⁴⁴ Jour. Infect. Dis., 1911, 9, p. 251.

⁴⁵ Jour. Am. Med. Assn., 1903, 41, p. 1009.

⁴⁶ Berl. klin. Wchnschr., 1913, 1, p. 517.

⁴⁷ Brit. Med. Jour., 1923, 21, p. 677.

but in relatively small numbers. These forms were designated more or less interchangeably as *B. putrificus* and *B. oedematis maligni*. Mace, quoted from Passini, found *B. oedematis maligni* to be a constant inhabitant of the normal large intestine, but Passini⁸ was unable to substantiate this. Metchnikoff¹³ isolated on occasions *B. sporogenes* from the stools of healthy persons, but states, in agreement with Retter and Herter, that proteolytic anaerobes are far more numerous in the stools of persons suffering from a "putrefactive condition of the intestine."

The most complete study of the fecal flora of normal persons was made by McNeal, Latzer and Kerr.⁴⁸ They found free spores almost always present in considerable number. These observers recognized 6 different types of anaerobic bacteria: (1) *B. welchii*; (2) essentially the same as (1), but forms spores more readily; probably *B. welchii* also; (3) does not hemolyze blood but produces green coloration about the colony on blood plates; (8) related to *B. oedematis maligni*, but is not pathologic for laboratory animals; rapidly digests milk casein; (9) resembles type 8 very closely but differs in the slower liquefaction of gelatin; (18) morphologically distinct from the others, forming a large, oval, terminal spore much wider than the bacterial cell. It resembles *B. putrificus* closely.

Of 139 stools examined for the presence of sporulating anaerobes, they recognized their type 1, 113 times; type 2, 25 times; type 3, 25 times; type 8, 14 times; type 9, 8 times, and type 18, 12 times.

From the meager culture evidence afforded, it is difficult, with the possible exception of *B. welchii*, to ascertain to what species the forms isolated belonged. The number of colonies appearing on the plates made by McNeal, Latzer and Kerr, were exceedingly variable, extending from 64,000 to 0 per mg. of feces. No tests were made to determine whether or not any of these colonies were formed by aerobic spore-bearing bacteria, which we now know might well be the case.

These observers conclude that *B. welchii* is a normal inhabitant of the intestine of man, and that the occurrence of other forms does not necessarily indicate a pathologic condition.

Hewlitt²² and Passini⁴⁹ isolated *B. welchii* from the stools of normal adults with considerable regularity. Orton,⁵⁰ after an exhaustive investigation, found *B. welchii* in the feces of 83.3% of the inmates of the Massachusetts State Hospital who had no digestive disturbance, and in 70% of normal persons outside of the hospital. Andrews⁵¹ mentions *B. welchii* as the most abundant anaerobe in the intestines of man. Simonds'⁵² observations entirely agree with those of Passini, McNeal, et al., Orton, Andrews and others, who find few fecal specimens of adults not containing this widely studied anaerobe.

The following summary from Mathews,⁵² although he does not specify anaerobic bacteria, serves admirably to express the popular but none too scientific views now prevalent concerning the rôles played by proteolytic micro-organisms in the intestine and certain pathologic conditions.

It is, however, from the bactericidal decomposition of the proteins that the most toxic substances are produced. The amino acids of the proteins set free by the intestinal enzymes are physiologically quite inert. They are absorbed and serve as foods. If they are introduced directly into the blood they cause no reaction. The bacteria, however, like the cells of the body,

⁴⁸ Jour. Infect. Dis., 1909, 6, p. 571.

⁴⁹ Centralbl. f. Bakteriöl., 1903, 32, p. 647.

⁵⁰ Jour. Med. Res., 1913-14, 29, p. 287.

⁵¹ Brit. Med. Jour., 1913, 1, p. 539.

⁵² Physiological Chemistry, 1920.

have the power of tearing these amino acids to pieces and some of these products are very toxic. They have the power of decomposing amino acids, setting free ammonia and leaving the fatty acids. Some of the products elaborated produce vasodilation, and lower the coagulability of the blood. By the decomposition of cysteine and cystine, hydrogen sulphide is formed. This is readily reabsorbed and produces headaches and depression. When absorbed in small quantities it is presumably one of the factors contributing to the anemia of those having chronic constipation. Mercaptans, i. e., methyl or ethyl sulphide, may also be formed, and these are very toxic. From tryptophane we have the toxic substances indole and skatole elaborated.

The putrefactive processes in the intestines also have a very remarkable relation to the skin. In nearly all cases of excessive intestinal putrefaction the whole organism has its vital resistance lowered. Pimples, pustules, acne and boils are constantly forming. The spotted skin is generally but not always a sign of intestinal putrefaction. Cold sores, increased sensitivity of the skin to infection, colds may develop, catarrh becomes worse, erythema may develop and various parts of the body become inflamed.

It is without the scope of this study to allude to the possibility of aerobic, nonspore-bearing bacteria in the intestine as possible causative agents of some of the untoward conditions indicated by Mathews. Some are of the opinion that those species having proteolytic powers, as well as others, may be the etiologic agents of a number of symptoms more or less well defined.

From the investigation about to be reported, we feel, however, that the significance of the anaerobic spore-bearers, with the possible exception of *B. welchii* and allied forms, is not as poignant as heretofore assumed.

TECHNIC

A detailed description of the technic, formulas for mediums and methods in general for cultivating and manipulating anaerobic spore-bearing bacteria will be found in an investigation recently reported by me.⁵³

The technic about to be given was found to be satisfactory for recovering anaerobic spore-bearing bacteria from human feces, and gives one an acceptable idea of the predominating types. I do not claim, however, that species which have occurred sparsely have always been successfully isolated in the cases reported.

Clean sterile glass or porcelain jars were usually supplied as containers, and the patient or attendant was cautioned to bring the specimen of feces to the laboratory as soon after defecation as possible. The stools were usually examined immediately on their receipt. If some delay was encountered, the specimen was kept at icebox temperature. Specimens which arrived very late, or for one reason or another had to be kept over night, were not examined. As complete a history as possible was obtained from the attending physician.

1. About 500 mg. of a well mixed specimen were emulsified in 9 c.c. of physiologic salt solution and heated in a water bath to between 80 and 82 C. for 15 minutes. It was found important to watch the specimen carefully while it was undergoing the heating process in order to keep the temperature at

⁵³ Jour. Med. Res., 1922, 43, p. 155.

the desired point. The heating, which is best carried on in a narrow test tube (15 x 1 cm.) to insure penetration, was, of course, employed to kill the aerobic nonspore-bearing forms.

Numerical Estimation.—Twelve test tubes, each containing 9 c.c. of 1% casein digest agar, were boiled for 15 minutes and then rapidly cooled to 45 C. Successive dilutions were made into these tubes by inoculating 1 c.c. of the fecal emulsions into the first tube, mixing thoroughly, inoculating 1 c.c. of the contents of tube 1 into tube 2, and so on, until finally all 12 tubes were seeded in graded dilution. Anaerobic conditions were obtained by applying a seal of sterile petrolatum some three-quarters of an inch high. The necessity of thoroughly mixing each tube of medium with the inoculum cannot be over-emphasized. The tubes were then placed in the incubator at 37 C. for 3 days.

I have found it highly impractical to attempt a direct quantitative estimation of the number of anaerobic spore-bearing bacteria contained in a given unit of fecal emulsion. This was due to a number of factors.

1. The stool specimens varied widely as regards their consistency. Some were fluid, some pasty, while others were solid. Some of them varied from solid to fluid in the same specimen. The specimens were, of course, thoroughly mixed, but it was felt that as regards the difference between the frankly fluid and solid specimens that a larger number of bacteria were to be expected where there was more solid fecal material per unit of measurement. That such a divergence did exist was noted by McNeal, Latzer and Kerr.⁴⁸

2. Although finely calibrated pipets were used and the technic controlled as carefully as possible, there was not always a diminution of the number of bacteria in the tubes as the dilution factor increased; that is to say, tube 4 would at times contain as many colonies as tube 3. This condition was, of course, probably due to a small particle of fecal matter being taken over into tube 4 with the fluid, such a particle being heavily loaded with bacteria. Whatever the cause, such discrepancies did occur and caused serious interference with the results of direct numerical calculation.

3. Occasionally no growth would occur in any of the tubes of agar planted, but the gram-stained film attested to the presence of spore-bearing bacteria. In such instances it was found necessary to cultivate the fecal emulsion primarily in Robertson's cooked meat medium and subsequently subcultivate into the 12 agar tubes. Such a procedure would give entirely erroneous numerical calculations as far as the concentration of bacteria in the original specimen is concerned. As a matter of routine, two tubes of cooked meat medium were inoculated with 1 c.c. each of the heated fecal emulsion—one tube to guard against such emergencies as just described, and the second to gain an idea of the potentialities of the anaerobic flora, whether strongly proteolytic, weakly proteolytic, or nonproteolytic. More of this will be said later.

The concentration of the bacteria in the specimen was usually estimated after 3 days' incubation. On the basis of the foregoing, it was found most feasible to make 5 subdivisions for this purpose: Group 1: No growth in agar tube 1 after 7 days; no growth in cooked meat medium after 7 days; no anaerobic spore-bearing bacteria present. Group 2: agar tube 1 not showing growth after 3 days' incubation, but growth in tube of cooked meat medium; very few. Group 3: growth in agar tubes 1, 2 and 3 after 3 days' incubation, but not in tubes of higher dilutions; few. Group 4: growth in agar tubes 1 to 8 inclusive after 3 days' incubation, but not higher; numerous. Group 5: growth in all agar tubes after 3 days' incubation; very numerous.

Tubes showing the best distribution of the colonies were selected for the purpose of isolating in pure culture and identifying the bacteria.

The tubes were thoroughly washed off with 1% lysol solution and dried with a piece of sterile cotton. A file mark was made with a hot file just at the junction of petrolatum and agar, and by smartly tapping the upper part of the tube it could usually be broken at that point. The broken tube was well flamed at the opening and the contents expelled into a sterile Petri plate.

Fishing Colonies.—Several sterile Pasteur pipets were made from soft, small bore glass tubing (15 x 0.1 cm.), and these were rested on a wire basket which had previously been sterilized by passing the flame of the Bunsen burner over it several times. It is best to make the tips of the pipets rather wide by snipping off the fine end with sterile forceps.

By elevating the Petri plates containing the shake cultures until they are directly in front of the open end of a Leitz microscopic desk lamp, I found that the colonies stand out in bold relief and thus successful picking of the colony is much facilitated. It is generally a simple matter to insert the tip of one of these fine pipets directly into the colony desired without coming in contact with neighboring ones. By applying gentle suction, one may usually pick up the entire colony in the Pasteur pipet.

The colony is then expelled into one of several tubes of 0.5% casein digest agar which have previously been boiled and are at hand in a water bath cooled to 45 C. This procedure was continued until several colonies were secured from each case for purposes of subsequent identification and further purification. But little reliance has been placed on colony morphology as an aid in differentiation, so that no study of the colony was made at this point with that end in view.

The cultures were next rendered anaerobic by applying a cap of sterile petrolatum some three-quarters of an inch high and incubated at 37 C. for 72 hours. Gram-stained preparations were then made to determine morphologic differences.

Test for the Presence of Aerobic Bacteria.—This was accomplished by inoculating a small amount of the foregoing soft agar cultures on slants of 1.5% casein digest agar, P_H 7.2. The slants were allowed to incubate for 72 hours, and if no growth appeared, the cultures were declared free of aerobic contamination. It was rather surprising to ascertain how infrequently these aerobes did occur. If their presence were detected, the cultures were reheated at 80 C. for 15 minutes and reinoculated into 0.5% casein digest agar that contained, in addition, gentian violet in final dilution of 1:400,000. The use of gentian violet for rendering cultures of anaerobic bacteria free from contamination, especially with the aerobic spore-bearers, was first pointed out by Hall,⁵⁴ who, following Churchman's⁵⁵ discovery of the selective bacteriostatic action of this dye, applied it to such purpose.

Plants were then made from the gentian violet agar to plain casein digest 0.5% agar. Such treatment usually rendered the cultures free from spore-bearing aerobes; but on several occasions the procedure was repeated as an added precaution. Successive tests for the presence of aerobes were always made after each attempt to eliminate them. Aerobic spore-bearing bacteria were relatively infrequent contaminants, as were gram-negative types. The most bothersome and frequent contaminations were heat-resistant cocci.

⁵⁴ Jour. Am. Med. Assn., 1919, 72, p. 224.

⁵⁵ Jour. Exper. Med., 1912, 16, p. 2.

Purifying Cultures for Subsequent Identification.—Barber's⁶⁰ single cell technic or Chamber's⁵⁷ modification of it was wholly relied on as a method of finally purifying these cultures. No attempt at identification was made until each individual culture was so treated. In comparison to other less certain methods, these single cell technics were found to be not nearly as laborious as is often assumed, and the little extra time and trouble are more than compensated for by the assurance of working with absolutely pure strains. In treating with anaerobic bacteria, the necessity of painstaking efforts necessary to obtain absolute purity of culture, while obvious, is not fully appreciated by those less familiar with this group. In addition to the possible contamination with aerobic micro-organisms, it has been my experience that even when cultures of anaerobic spore-bearers are purified by the deep culture method of the French observers, Veillon and Zuber,⁵⁸ Weinberg and Seguin,⁵⁹ and others of this school, or by the surface colony method used widely in this country and England, single colonies are sometimes encountered composed of anaerobes of more than one species. I consider this peculiar feature of chance juxtaposition responsible for much of the confusion which has existed in anaerobic bacteriology up to very recent times. Suffice it here to say that no attempts at differentiation were made until cultures from single colonies were first purified by either Barber's single cell method or that of Chambers.

Chambers' micromanipulator has been found serviceable for isolating single bacterial cells, as I have reported, giving also a short description concerning its application to this purpose⁶⁰

Barber⁶¹ found that single free spores or spore-bearing bacteria of the anaerobic group give by far a larger number of successful cultures than do single vegetative forms. This was also my experience. When it was found necessary to induce the development of the spore stage, cultures were planted into sugar-free casein digest broth, P_H 7.2. These tubes were incubated for about 18 hours, for it was found best to work with cultures as young as possible when isolating single cells. *B. welchii* and other members of the nonproteolytic group often tenaciously refused to sporulate in the medium mentioned above. In such instances, single bacterial cells were isolated from very young cultures giving only the faintest perceptible cloud to the broth, usually from 4 to 8 hours old. As a matter of routine, 10 single cells were isolated from each cultured colony. Each cell was planted into a tube of 0.5% casein digest agar, P_H 7.2, previously boiled for 15 minutes and standing in a water bath cooled to 45 C. The tubes were rendered anaerobic by applying a seal of sterile petrolatum and incubated for 4 days. Those not showing growth at the end of this period were incubated 3 days longer. After 7 days, the apparently sterile tubes were discarded. It was unusual to have a series without a single successful plant. When such was the case, the process was repeated until cultures of one cell were obtained. Ordinarily, 3 successful isolations were obtained from 10 plants, and not infrequently 4 or 5. On two occasions, 7 successful inoculations were recorded from 10 plants. Both of these proved to be profusely sporulating strains of *B. sporogenes*.

A separate account of the positive single cell cultures was kept for each colony, and each positive culture was subsequently individually identified. In

⁵⁶ Philippine Jour. Sc., 1914, 9, p. 307.

⁵⁷ Jour. Bacteriol., 1923, 8, p. 1.

⁵⁸ Arch. de med. exper. et d'anat. path., 1898, 10, p. 517.

⁵⁹ La Gazeuse Gangrene, 1917.

⁶⁰ Kahn: Jour. Infect. Dis., 1922, 31, p.344.

⁶¹ Jour. Exper. Med., 1920, 27, p. 240.

this way, an idea was gained of the relative frequency of a given species when more than one occurred in an individual case. For instance, if there were present what afterward proved to be 1 colony of *B. sporogenes* and 3 colonies of *B. aerofoetidis*, even though the 1 colony of *B. sporogenes* gave a much larger series of single cell positive plants, if it were known that 3 colonies of *B. aerofoetidis* were acted on, it could safely be assumed that *B. aerofoetidis* was the spore-bearing anaerobe in the majority. To supplement this, however, a careful study was made of the proteolytic characteristics of the anaerobic spore-bearing bacterial flora of each case.

Proteolytic Action of Total Anaerobic Bacterial Flora Spore-Bearing from Each Case.—I desired to ascertain whether the predominating characteristics of this part of the flora were strongly proteolytic, feebly proteolytic, or non-proteolytic. To accomplish this, 1 c.c. of heated fecal emulsion from each of these cases was inoculated into cooked meat medium that had been previously boiled and cooled. Holman⁶² made a suggestive study of the action of certain bacteria on cooked meat medium and found, as I did, that changes in reaction, color of the meat, and evidence of varying degrees of putrefaction of the meat make it a medium of great importance in aiding the differentiation of certain of the anaerobic species. Besides this differential value, he also found that the most favorable characteristic, aside from its growth-stimulating properties, is the buffer influence exerted. The products of growth, namely, the acids of the nonproteolytic species, are neutralized up to a certain point so as not to interfere markedly with the characteristic growth of a predominating proteolytic type, if such be present. On the other hand, if members of the nonproteolytic group are present in the majority, the buffer characteristic of the cooked meat medium is overcome, and the saccharolytic types are able to assert themselves and give rise to a typical reaction.

In studying the potentialities of the combined anaerobic spore-bearing flora, 5 types of reaction were recognized:

1. Typical *B. welchii* Reaction: The gas production is usually very copious, at times so marked as to suggest gentle boiling. The petrolatum cap is often blown from the tube. The meat is almost scarlet in color or deep terra cotta. No proteolysis is evident after 3 or 4 days' incubation. Although if types belonging to the strongly proteolytic group are secondarily present in considerable number, there is evidence of digestion of the meat on the 4th or 5th day. Such activity may become marked after 7 days' incubation. Such a reaction strongly suggests that the anaerobic spore-bearing flora is composed of *B. welchii* in large majority.

2. Reaction When Anaerobic Bacteria of Nonproteolytic Group, Other Than *B. Welchii*, Are Present in Numerical Superiority; *B. Fallax*, *B. Tertius*, Etc.: Gas production is usually copious, although varying. Meat assumes a much lighter terra cotta color, often nearing salmon pink. There is no evidence of digestion of the meat after 2 or 3 days' incubation. After that time, however, if *B. welchii* be present in considerable numbers, the meat may assume a deeper red color. If proteolytic species are appreciably secondary, the meat after the 3rd or 4th day will show evidence of digestion, foul odor, darkening and some decrease in volume.

3. Reaction Characterized by a Predominance of Feebly Proteolytic Anaerobic Spore-Bearers, *B. Putrificus*, *B. aerofoetidis*, Etc.: Gas production is feeble to marked. The meat is darkened somewhat, but not actively digested;

⁶² Jour. Bacteriol., 1919, 4, p. 282.

the odor is rather foul. This occurs after 2 or 3 days' incubation. If bacteria of the strongly proteolytic group be secondary, digestion of the meat with marked blackening ensues after 4 or 5 days. At this time also gas production may increase. Nonproteolytic types do not here visibly assert themselves in secondary reaction.

4. Reaction Characterizing Predominance of Strongly Proteolytic Anaerobic Spore-Bearers, *B. Sporogenes*, Etc.: Gas production is usually marked, but varying. As a rule, it is not as copious as in the reactions previously described. The meat is markedly darkened after 24 hours. The odor is foul and penetrating. Digestion of meat is rapid, especially after 48 hours. The broth remains tenaciously clouded. The presence of secondary types is completely masked by this strongly proteolytic picture.

5. Reaction Characterized by a Predominance of *B. Bifermentans*: Gas is produced in varying amounts, usually not marked. The meat is darkened, at times assuming a mottled appearance produced by alternate areas of brownish-red and black. The odor is foul but not as penetratingly so as that produced by *B. sporogenes*. Digestion of the meat ordinarily is not marked. If *B. sporogenes* is present and numerically secondary, proteolysis may be more pronounced, especially after 5 days. Digestion of the meat is never as marked as in cultures composed principally of *B. sporogenes*. Nonproteolytic anaerobes do not assert themselves in this type of reaction.

Some may be of the opinion that a tube of cooked meat medium does not approximate the intestinal environment very closely, and on that account the reactions just described would lead one to erroneous conclusions. This may or may not be true. Of course at the present time there is no exact way of ascertaining to just what extent anaerobic spore-bearing bacteria multiply within the intestinal canal, and, if such multiplication does go on, how their metabolism is influenced by diet, individual susceptibility, virulence of strains, interassociation of the various species of anaerobes, suppressing activities of certain aerobic types and the like. On the other hand, it is logical to assume that in a medium having the peculiarities attributed to the cooked meat substance, buffer influence, for instance, those anaerobes occurring in superior number will exert their characteristic reaction, at least as far as this substance is concerned. In this way, then, an idea is to be had of the predominant type present in the intestine. I claim no more. In the following sections, it will be seen that the reactions taking place in the cooked meat tube approximated closely those characteristic of the types of anaerobic spore-bearers which later were found by more exact measures to be predominating. For this reason I feel that for those not having the time nor training necessary for the study of the intestinal anaerobes by more detailed methods, a good deal of information may be gleaned from reactions taking place in tubes of cooked meat medium.

It is quite possible that some of the species occurring infrequently in the culture tubes were present in relatively greater abundance within the intestine. It is also possible that some types have been missed altogether, failing for one reason or another to multiply on artificial mediums or out of the intestinal environment. I feel, however, that the possibilities of these conditions are relatively remote. The medium used by me has proved, time and time again, able, with suitable anaerobiosis, to bring to development the most fastidious and delicate species as regards their nutritive and oxygen requirements. Such micro-organisms as *B. oedematiens*, *B. aerofoeditis*, and *Vibrio septique* have been isolated from the feces in actual cases, indicating that the technic used was quite applicable to the problem.

As regards quantitative estimation, I do not claim to have been able to ascertain with numerical accuracy the relative percentage of occurrence of the various anaerobes in a single case, nor the actual numbers of a single species if it alone were present in the given weight of fecal material examined. It is highly practical, however, to determine with this technic the relative frequency of occurrence of several species within a single case, at the same time maintaining absolute purity of culture. It is practical also to estimate the degree of infectivity of the various cases; that is to say, whether they were anaerobe free, contained very few, few, numerous or very numerous bacteria of the anaerobic spore-bearing group.

The most pronounced difficulties pertaining to accurate quantitative and qualitative estimation of anaerobic spore-bearing bacteria are to be found in the little differential aid afforded by the morphology of the micro-organisms or the colonies formed by them. Among recent investigators, Henry,⁶³ Weinberg and Seguin,⁶⁴ The British Medical Research Committee⁶⁴ and Heller,⁶⁵ have placed considerable emphasis on these points as being of diagnostic value. I have found, as has been reported, that morphology, especially that of the individual colony, offers very little aid in telling one species of anaerobe from another, and is, to my mind, a wholly unreliable, hazardous and confusing method of grouping. Hall,⁶⁶ in his careful study of the anaerobes, found colony formation to be variable within a species for a number of the different types. Kendall, Day and Walker,⁶⁷ in describing some of the members of this group, gave no space to the size and shape of the colony. I am in hopes that attempts to differentiate by this method will soon no longer be attempted.

Anaerobiosis was induced in all cultures by boiling the tubes for 15 minutes, rapidly cooling to 45 C., inoculating and then applying a cap of sterile petrolatum about $\frac{3}{4}$ inch in height.

CLASSIFICATION AND DIFFERENTIATION OF THE ANAEROBIC SPORE-BEARING BACTERIA ISOLATED FROM CASES

At the outbreak of the Great War, the much needed impetus was furnished to prompt investigators to a careful study of this group of bacteria. This was due to their close association with gas gangrene and gunshot wound infection. As has been pointed out on numerous occasions, prior to that time our all too superficial knowledge of the anaerobes was limited to such forms as *B. tetani*, *B. botulinus*, the somewhat phantasmal *Vibrio septique*, *B. welchii* and *B. sporogenes*. The latter two species were known interchangeably under many different names. Since then, more certain cultural characteristics have been established for these bacteria, and 14 other definite species have been added to the list of known anaerobes. Bacteriologists are still in debate over the identity of certain others.

⁶³ Jour. Path. and Bacteriol., 1916, 20, p. 327.

⁶⁴ Reports of the British Medical Research Committee on Anaerobic Bacteria, 1915, 1916, 1918, 1920.

⁶⁵ Jour. Bacteriol., 1921, 6, p. 461.

⁶⁶ Jour. Infect. Dis., 1922, 30, p. 141.

⁶⁷ Jour. Infect. Dis., 1922, 30, p. 141.

The confused conditions in which information of this group existed may now be traced to several factors. The foremost and outstanding of these are: (1) impurity of culture caused by a lack of adequate technic of making culture and purifying; (2) the failure to recognize the ease with which cultures of anaerobic bacteria are contaminated with other anaerobes, especially *B. sporogenes*; (3) the most subtle of all difficulties confronting workers with these micro-organisms, namely, the frequent occurrence of colonies containing more than one species.

The literature abounds with instances in which cultures painstakingly investigated and described as a single species, are, in the light of present-day information, known to have contained 2, and sometimes 3, distinct types. A typical illustration of this kind of error may be had from Klein's⁶⁸ description of his *B. enteritidis sporogenes*, for which powerful saccharolytic and proteolytic properties were attributed. Welch and Nuttall primarily isolated *B. welchii* from a culture of Klein's bacillus, supposedly pure, and *B. sporogenes* has also been recovered from a similar source. Koch described at great length his *B. oedematis maligni*, and his controversy with Pasteur over the probable identity of this "species" and that of Pasteur's *Vibrio septique*, kept investigators in doubt for a number of years. It is now agreed that Koch's culture was composed principally of *B. sporogenes*, a nonpathogenic species, masking what, if any, disease-producing bacteria there may have originally been present.

It is difficult to praise too highly the contributions of Muriel Robertson and the Medical Research Committee working in England; the untiring efforts of the French investigators, Weinberg and Seguin, whose monograph on the bacteriology of gas gangrene laid the foundation of present-day anaerobic bacteriology; or the efforts of the American investigators, Herter, Rettger, Reddish, Simonds, Hall, Heller, Kendall and his co-workers. Gradually the bacteriology of the spore-bearing anaerobic bacteria has come to assume an ordered condition.

Early in 1919 when this study was first undertaken, much of the present-day information on the anaerobes had not been published. On going over the then existing literature, I became much impressed with the necessity of devising adequate means of growing and purifying cultures of the several different species, and studying in detail their cultural and differential features, in order to have a standard of comparison to ascertain the intestinal types which were, and would be,

⁶⁸ Centralbl. f. Bakteriöl., 1895, 18, p. 737.

subsequently isolated from the stool specimens. Methods were cast about for which would decide these questions once and for all. Several months were devoted to the study of various technics then described. For the most part, these were discarded as unsuitable for the purpose involving the systematic study of a large number of species, as some of the types refused to grow with regularity, or the old pitfall of impure colony was encountered. This led to the use of the single cell technic for the purpose of purifying, together with more suitable culture mediums, with much more satisfactory results. In 1922, a detailed report was made of the cultural and differential features of 15 anaerobic spore-bearing bacteria of various species, all of which had been grown from single cells. I may add that this study was undertaken with the express intent of formulating a series of comparative standards for the present investigation.

In 1922, also, there appeared the results of a series of experiments carried on by Kendall, Day and Walker⁶⁷ on the chemistry of the metabolism of this group, containing, in addition, considerable cultural information. Shortly after the publication of our own study, the careful investigation of Hall⁶⁸ appeared, embracing about the same scope. Reddish and Rettger⁶⁹ published a preliminary account of the cultural features of certain members of this anaerobic group, but the detailed account of their excellent investigation did not appear in the literature until recently.

In the main, these later American investigators seem to agree on the more salient differential cultural features of these bacteria, but there still does exist, on the other hand, some divergence of opinion concerning diagnostic characteristics less pronounced. Among these are: (1) some of the fermentative reactions, (2) the use of morphology as a differential criterion, (3) the proteolytic activities of *B. bifermentans*, (4) the production of tyrosine by some of these bacteria.

Stimulated by the apparent differences in result concerning the foregoing points, I have undertaken, from time to time, a reinvestigation of some of the topics in question, with further conclusions as follows:

1. *Fermentative Reactions*.—Those in question have been found to be as initially reported, with the exception of *B. tetani*.

In studying anaerobes of the same species isolated from other sources, however, one may expect to encounter some slight latitude of variation as regards fermentation of the various carbohydrates used for

⁶⁹ Abstr. Bacteriol., 1922, 6, p. 7.

differential purposes. That is to say, the degree of acidity encountered when certain sugars are attacked may not always be reduced to exactly the same end point, i. e., P_H 6.2, for instance. At certain times this variation ranges from P_H 6.8 to P_H 6.4, carbohydrate, mediums, species of anaerobe, concentration of the sugar and initial degree of acidity of the medium all being constant. These variations may be accounted for, to some extent at least, by the ability of certain strains of the same species to attack the carbohydrate in question with greater or less energy than do certain others. This point has been considered carefully during the process of classification.

Although the fermentative characteristics of the group form the most reliable single basis of classification, there is no doubt that other cultural features must be taken into consideration as well, before valid results may be obtained.

This involves a careful scrutiny of the proteolytic activities, pathogenic properties, morphology to some extent; also, at times, the hemolytic activities of closely related species will be found to aid materially.

Regarding the use of acid or gas production as the safer criterion of actual carbohydrate fermentation, in agreement with Hall,⁶⁶ a measurement of the production of acid is by far the most reliable. If one depends on the measurement of gas alone, false results will surely be obtained. I have noted on several occasions that cultures producing a moderate amount of gas (this is especially evident as regards *B. putrificus*) may give rise to little or no alteration in the concentration of the H-ions. On the other hand, cultures showing traces only, or at times no visible evidence of gas production, will be found to have elaborated a considerable quantity of acid. It is most important, then, to take the H-ion concentration produced by each culture when studying the fermentative reactions of this group. The colorimetric method of Clark and Lubs⁷⁰ is entirely suitable for this purpose.

The variations encountered as regards *B. tetani* will be discussed under the heading of the individual species.

2. *The Use of Morphology as an Aid in Classification.*—In a recent study of the spore-bearing anaerobes, I reported finding the use of morphology an unreliable method for differentiating species belonging to this group of bacteria. This statement should now be modified somewhat.

When one desires to differentiate the so-called subterminal sporulating anaerobes from those having the spore centrally situated, it is

⁷⁰ Jour. Bacteriol., 1917, 2, p. 1.

my opinion that it indeed would be a hazardous venture to place much credence on the position of the spore or the general morphology alone of the bacteria in question. In pure cultures of *B. bifermentans*, a central sporulating anaerobe, according to most investigators, a considerable number of individuals have been observed by me possessing subterminal spores. *B. sporogenes* gives rise, usually, to subterminal sporulating bacteria, but those having the spore placed centrally are by no means uncommon. Still attempts are frequently made to classify these types on the position of the spore. It seems that terminally sporulating types may, with a fair degree of accuracy, be differentiated as a group from those species having the spore situated centrally or subterminally, but final grouping can be made only after other, and much more important, separative factors have been taken into consideration. It is to be remembered that anaerobic spore-bearing bacteria, as far as their morphology is concerned, seem to be susceptible to change in environment, i. e., culture medium employed, degree of anaerobiosis afforded, age of culture and the like. In a study of this kind, then, in which a considerable number of species are often being classified in a single case, the gram-stained film offers only the most superficial kind of information.

3. *Production of Tyrosine by Some of the Bacteria of the Proteolytic Group.*—The cultures of *B. sporogenes* isolated from these cases are found to produce tyrosine as originally reported for the single cell standards. The same is true concerning cultures of *B. putrificus* and stock cultures of *B. botulinus*, the latter producing considerably much less than does *B. sporogenes*. Cultures of *B. bifermentans* isolated have not produced tyrosine, as far as I am able to tell, on any of the mediums designed to show proteolysis. These results differ from those obtained by Hall,⁶⁶ who finds *B. bifermentans* to be a tyrosine producer but not *B. sporogenes*, *B. putrificus* or *B. botulinus*. Hall⁷¹ recently described an organism for which he proposes the name "*B. tyrosinogenes*." The physiologic properties attributed to this species are identical with those of *B. sporogenes*, except that, according to Hall, the former produces tyrosine, but not the latter. This subdivision is of doubtful value, as it is highly probable that Hall is here working with a strain variation and that no subdivision should be made. In our strains of *B. sporogenes* there is considerable degree of variation as regards the amount of tyrosine produced, especially in young cultures,

⁷¹ Abstr. Bacteriol., 1921, 2, p. 6.

the substance being elaborated much more abundantly in some cultures than in others. In all old cultures of *B. sporogenes*, grown in cooked meat medium for 2 weeks or more, the characteristic wheat sheaf-like bundles of tyrosine crystals are to be noted. Some of these were kindly identified chemically by Dr. Ellis M. Black, late of this institution.

My observations on the elaboration of tyrosine for the following organisms are in accord with those of Hall: Nonproducers of tyrosine: *B. welchii*, *B. oedematiens* (*B. novyi*), *Vibrio septique*, *B. teritus*, *B. tetani*, *B. butyricus*, and *B. tetanomorphus*, *B. aerofoetidis* and *B. fallax*. Producers of tyrosine: *B. histolyticus*.

In my primary study of the differential features of the spore-bearing anaerobes it was stated that since the results reported were derived from observations made on 1, or at most 4, cultures of a single variety, no claims were made to the effect that the cultural reaction given would be exactly constant for all representatives of a given species. Such, to a limited extent, has been the case. At times, strains have been encountered which differed more or less from the original single cell standards. At times, these differences were also observed in the reports of other investigators. Since the identification of the 20 species here considered is by no means, even now, a scientific certainty, I have not hesitated when discrepancies arose to consult freely the work of others interested in the group. The reports of Weinberg and Seguin, Hall, and Kendall, Day and Walker have given material aid on more than one occasion. When the results obtained by others were diametrically opposed to mine, I have relied on observations made from the single cell standards.

In view of these differences of opinion, it is necessary to show just how the cultures isolated from the cases were grouped and identified. For that reason, there will be found in the following paragraphs a compilation of some of the historical and outstanding cultural features of these bacteria, which subsequently led to their identification.

OUTLINE OF PROCEDURE IN CLASSIFYING ANAEROBIC SPORE-BEARING BACTERIA

1. Isolation with Single Cell Apparatus.
2. Proteolytic Tests:
 - (a) Cooked meat medium.
 - (b) Milk.
 - (c) Gelatin.
 - (d) Coagulated egg albumin in meat infusion casein-digest broth.
 - (e) Inspissated serum.
3. Subdivision into one of the Following Groups:
 - (a) Strongly proteolytic: *B. sporogenes*, *B. centrosporogenes*, *N. Sp.* (Hall), *B. botulinus*, *B. histolyticus*.

- (b) Feebly proteolytic: *B. bifermentans*, *B. putrificus*, *B. tetani*, *B. tetanomorphus*, *B. aerofoetidis*.
- (c) Nonproteolytic: *B. welchii*, *B. tertius*, *B. fallax*, *B. oedematiens* (*B. novyi*), *Vibrio septique*, *B. chauvœi*, *B. butyricus*, *B. sphenoides*.
- 4. Fermentation Tests: Carbohydrates Used: Glucose, levulose, galactose, saccharose, maltose, lactose, mannose, xylose, starch, inulin, dextrin, salicin and glycerol.
- 5. Morphology: Gram Stained Preparation.
- 6. Hemolytic Tests (when conditions warranted) Lyall's Technic.⁷²
- 7. Pathologic Tests (when conditions warranted).

OUTLINE OF THE HISTORICAL FEATURES AND CULTURAL CHARACTERISTICS
OF THE ANAEROBIC SPORE-BEARING BACTERIA

B. sporogenes (Metchnikoff).⁷³ This is probably one of the most actively proteolytic bacteria known. In fact, it was this dominant cultural characteristic which led to its isolation from human feces. It is widely distributed in nature and a frequent and bothersome contaminator of other cultures of anaerobes. It is relatively a large gram-positive bacillus, usually forming subterminal spores in great profusion on practically any kind of cultural medium. Free spores are formed in abundance. Contrary to the opinion of Hall⁶⁶ and others, I find central sporulating forms by no means uncommon. This organism is a member of the strongly proteolytic group. On cooked meat medium, egg albumin cube broth, milk, inspissated serum, the pabulum is rapidly darkened and digested. Gas production accompanies this proteolysis in varying amounts, and a pronounced, penetrating foul odor. Gelatin is markedly darkened and liquefied. Indol is not produced. Glucose, levulose, galactose and maltose are actively fermented, with acid and gas production. Lactose is not fermented. Glycerol is feebly attacked by some strains (P_H 6.6), but this is not now taken to be a cultural feature. Kendall, Day and Walker⁶⁷ consider glycerol fermentation one of the cultural characteristics of this species. Hall does not. Methemoglobin is elaborated by all strains tested by me.

In pure culture, *B. sporogenes* is absolutely nonpathogenic for laboratory animals. We have tested several strains isolated from intestinal sources on white rats and guinea-pigs, giving intraperitoneal injections of 2 c.c. of a 24-48-hour broth culture, without any evidence of effects. Hall tested this species on guinea-pigs and rabbits by subcutaneous and intravenous injection. He observed no effects from intravenous injection, and produced with subcutaneous doses only a slight tumefaction, which dried up and disappeared in a few days. On the other hand, Bargert and Dale⁷⁴ produced, with larger doses of old putrefactive cultures of this species, anaphylactic-like symptoms which appeared after the inoculation of small dosage into laboratory animals. Hall does not ascribe these to a pathogenic action of the micro-organism or to a specific toxin as is the view of Weinberg and Seguin,⁶² but rather to the effect of ptomaine-like substances produced during growth on protein-rich mediums.

The metabolic characteristics of *B. sporogenes* have been investigated thoroughly by Wolf and Harris,⁷⁵ Harris,⁷⁶ Kendall et al.⁶⁷ These observers

⁷² Jour. Med. Res., 1914, 30, p. 487.

⁷³ Ann. de l'Inst. Pasteur, 1908, 22, p. 930.

⁷⁴ Brit. Med. Jour., 1915, 2, p. 808.

⁷⁵ Jour. Path. and Bacteriol., 1916, 21, p. 386.

⁷⁶ Jour. Path. and Bacteriol., 1919, 23, p. 30.

found *B. sporogenes* capable of considerable ammonia production. Kendall found that amino nitrogen was produced in amounts twice that of ammonia production when this organism was grown in protein-rich medium, such as gelatin or milk. In medium containing protein of the peptone type, however, such as peptone water, the amino nitrogen content diminished incidentally to the growth of the organism. Ammonia was formed at about the same rate in either type of substance. They found that the addition of non-nitrogenous sources of energy, such as glucose, reduced markedly the formation of both amino nitrogen and ammonia. The Kendall group found a slight difference in the ability of some of their strains of *B. sporogenes* to produce ammonia and amino nitrogen, but consider this quantitative rather than qualitative.

B. sporogenes has been isolated with great frequency from the feces of the cases examined.

B. centrosporogenes (Hall).—This form was at first taken by Hall to be a variant of *B. bifermentans*. It differed from that organism, however, by its failure to agglutinate in a polyvalent *B. bifermentans* serum, in being motile and in some degree as regards colony morphology, "the colonies in 1% meat infusions agar being more fluffy and more diffuse than colonies of *B. bifermentans*."

The British Medical Research Committee⁷⁷ advocated the grouping of the "less frequent," highly putrefactive, motile anaerobes with central spores, under the heading of *B. sporogenes*. Hall states that 4 cultures of this type of bacteria, which he has studied, differ sufficiently to warrant a separate specific name. The main difference seems to be in the ability of cultures of *B. centrosporogenes* to produce white balls of tyrosine or leucine crystals in old deep brain medium cultures, and in the central location of the spore. In a single culture of *B. centrosporogenes*, kindly sent us by Hall, we have also noted these cultural features, but we cannot agree that they are absolutely distinctive. The production of tyrosine crystals is not an infrequent occurrence in old cooked meat medium culture of *B. sporogenes*, and forms with centrally located spores are more or less common. These observations were made on cultures of *B. sporogenes* grown from single cells, so that there can be no question as to their purity. In the culture received from Hall, however, there was a marked and constant difference in the growth cloud of *B. centrosporogenes*, the *B. centrosporogenes* type growing much less actively than *B. sporogenes* and giving rise to a marked mucoid-like deposit not noted in cultures of *B. sporogenes*. Hall, on the other hand, did not observe this feature in all cultures of his organism. Centrally located spores are much more numerous in this *B. centrosporogenes* culture. All other cultural, physiologic and pathogenic features of this species, as described by Hall, are identical with those of *B. sporogenes*. *B. centrosporogenes* is not pathogenic.

I have isolated from stools on 2 or 3 occasions an organism sporulating for the most part centrally; producing strong putrefaction on cooked meat medium, egg albumin cube broth, and other substances designed to show proteolysis; liquefying gelatin and showing carbohydrate fermenting properties similar to those of *B. sporogenes*. When, in addition to these cultural features, a marked mucoid-like product of growth was elaborated, the species was designated *B. centrosporogenes* in accordance with Hall.

It is quite possible that we are dealing with a strain variant of *B. sporogenes* and that the cultural features exhibited by Hall's type are not sufficient to warrant a new specific name. There can be no question, however,

⁷⁷ Medical Research Committee, Special Report, 1917, Series 12.

but that some minor differences are present. As serologic methods are, with one or two exceptions, unsuitable for differentiating anaerobic spore-bearing bacteria, it is just as logical to call the type in question "*B. centrosporogenes*" as to name it "Type B, *B. sporogenes*."

B. botulinus (Van Ermengen).⁷⁸—Kendall, Day and Walker⁶⁷ state that *B. botulinus* cannot be classed as a proteolytic species, while the Medical Research Commission⁷⁹ are inclined to group *B. botulinus* as one of the saccharolytic types, and place no emphasis on its proteolytic ability as an outstanding cultural feature. As regards this point, our results are diametrically opposed. We find *B. botulinus* practically as strong in its ability to digest protein as *B. sporogenes*, and in this view we are substantiated by Hall⁶⁸ who produced results similar to ours with all of his cultures of *B. botulinus*. As strong proteolytic ability is so outstanding a cultural feature of this species, it must be assumed that either Kendall and his co-workers and the British Medical Research Committee⁷⁸ were working with impure strains, or that the methods used by them for growing this species were entirely inadequate and did not give proper latitude of action to the protein-splitting enzymes.

B. botulinus, as is well known, produces a highly fatal neurotoxin, which, when swallowed, actively resists the destroying agencies of the gastro-intestinal tract. It therefore produces the only bacterial toxin that is fatal when fed, and is 1 of the 2 pathogenic members of the strongly proteolytic group of spore-bearing anaerobes, *B. histolyticus* being the other. These points are of outstanding and valuable diagnostic importance.

There are two well-known types of *B. botulinus*, viz., A and B. Recently Bengtson⁸⁰ isolated a 3rd representative of this group from the maggot of a fly which showed sufficient serologic and cultural differences to warrant her designating it "type C." Types A and B are by far the most common. Graham and Schwartz⁸¹ have shown that type A is more toxic than type B, when fed in the form of a 48-hour glucose broth culture to chickens.

Although slight morphologic and cultural differences do exist between *B. botulinus* and other similar types, the most reliable method of differentiating it is to inject 1 c.c. of a 24-hour broth culture of the organism in question, intraperitoneally, into a guinea-pig. If death occurs after a day, the culture is probably *B. botulinus*, provided purity and strong proteolysis.

B. botulinus is a large, thick, gram-positive bacillus with rounded ends. The spores, for the most part, are situated subterminally; a few are central. There is usually swelling of the rod at the point of sporulation. On stiff, sugar-free agar the vegetative forms are, on the whole, more slender than the spore-bearing types. The average size of this bacillus in the spore-forming stage is about $3.5 \times 0.6 \mu$. *B. botulinus* does not sporulate profusely.

This species is strongly proteolytic. It actively blackens and digests cooked meat medium, coagulated egg albumin and inspissated serum. Milk is clotted and then digested, leaving only a straw-colored fluid. Gelatin is quickly liquefied. Glucose, levulose and galactose seem to be attacked feebly, while the glycerol forms a more acceptable source of non-nitrogenous energy. In our studies of this species, maltose and starch were not utilized by the organism, nor was lactose. In these results we differ somewhat from those reported by Kendall and his associates, who claim that maltose and starch are fer-

⁷⁸ Centralbl. f. Bakteriöl., 1896, 19, p. 1.

⁷⁹ Brit. Med. Res. Com., 1919, No. 39.

⁸⁰ Rep. U. S. Pub. Health Service, 1922, No. 726.

⁸¹ Jour. Infect. Dis., 1921, 28, p. 317.

mented. These differences may be partially accounted for at least by the differences in methods used as actual criterion of fermentation. The Kendall group seemed to rely principally on the formation of gas, while I undertook to measure the H-ion concentration of the cultures in question. Gas production is not a reliable criterion of fermentation. In any event, carbohydrate fermentation does not form a reliable basis on which to differentiate *B. botulinus* from other members of the strongly proteolytic group. Greater stress should be placed on the production of the potent toxin. Methemoglobin is elaborated by *B. botulinus*.

In marked contrast to the work of Tanner and Dack,⁸² who claim to have found virulent toxin-producing forms of *B. botulinus* in 2 out of 10 stool specimens of normal, healthy persons examined by them, and who state also that 5 other stools gave strong evidence of the presence of this micro-organism, we, during the course of this study did not chance on a single culture of this species, although toxigenic tests were made on suspected cultures of *B. sporogenes* and *B. bifermentans*. It may be said that Tanner and Dack did not include in their article any cultural information in regard to the organisms isolated, nor did they give an outline of the technic used by them.

B. histolyticus (Weinberg and Seguin).⁸³—This peculiar and interesting species was isolated from a case of gas gangrene by the investigators named in the foregoing. It is the only member of the anaerobic spore-bearing group able to produce in living animals an extensive nonputrid type of progressive digestion of living tissue. One-half c.c. of a 24-hour broth culture of *B. histolyticus*, injected into the thigh muscles of a guinea-pig, will often, after 48 hours, cause necrosis of all of the muscular tissue, leaving only the bone. Such animals, on several occasions, have been found alive, and as far as could be determined, suffered no especial toxemia, as they were eating and apparently alert and active. In man, however, the presence of *B. histolyticus* in wounds is serious, and may lead to fatal toxemia.

Weinberg and Seguin have isolated a soluble toxin from cultures of *B. histolyticus* which they claim resembles that produced by *B. welchii*, *B. oedematiens* and *Vibrio septique*, although *B. histolyticus* does not resemble these species in any other respect.

B. histolyticus is gram-positive, and the spores for the most part are situated subterminally. It is a medium-sized organism, measuring, when grown in liquid medium, about $2.5 \times 0.7 \mu$. Swelling is often exhibited at the point of sporulation. On stiff, sugar-free agar, there is quite another picture; the bacilli are much smaller, do not stain deeply with the gentian violet, and forms having the spore situated terminally are not infrequent.

While slower and somewhat weaker in its proteolytic activities than *B. sporogenes* and *B. botulinus*, *B. histolyticus* surely warrants a place in the strongly proteolytic group.

Cooked meat medium is slowly, but finally, digested. Characteristic white balls of tyrosine are formed on this medium. The solidified egg albumin cube suspended in broth is attacked, but much less rapidly than is the case with the other members of this group. Milk is clotted and finally digested, leaving a straw-colored liquid. Inspissated serum is blackened, and slowly but completely digested. Most of these reactions take place without the production of gas; at most, only a bubble or so is to be seen. This is in marked contrast to the large amounts of gas evolved by *B. sporogenes* and *B. botulinus* when

⁸² Jour. Am. Med. Assn., 1922, 79, p. 132.

⁸³ Compt. rend. Acad. de sc., 1916, 163, p. 449.

grown in the same types of medium. The odor produced by *B. histolyticus* as a result of putrefaction is comparatively faint. Gelatin is rapidly liquefied. Glucose, levulose, galactose and saccharose are not attacked. There is no reduction in the H-ion concentration, nor is any gas elaborated. Acid may be produced in the presence of maltose, lactose and xylose, but there is no evolution of gas.

Hall claimed to have found that *B. histolyticus* produced acid and gas in the presence of glucose. After investigating strains that I sent him, he is convinced that such is not the case. Kendall and his co-workers found *B. histolyticus* to be nonfermentative. Weinberg and Seguin are dubious over the fermenting activities of this species, especially as regards the monosaccharides. Henry⁸⁴ claimed that his cultures of *B. histolyticus* fermented the monosaccharides, but states that his strains may have been impure. Wolf and Harris,⁸⁵ while studying the biochemistry of this species, found that the little gas produced was entirely elaborated from the protein metabolism.

I feel, therefore, that it is safe to take the lack of the ability of *B. histolyticus* to elaborate gas and acid in the presence of the monosaccharides, together with the characteristic, nonputrid, extensive style of necrosis produced when injected into laboratory animals, as typical outstanding features sufficient to differentiate this species from any other of the spore-bearing anaerobes. *B. histolyticus* is actively hemolytic.

B. oedematis maligni (Koch).⁸⁶—The organisms described heretofore as *B. oedematis maligni* are now taken to be identical with *B. sporogenes*.

B. bifermentans (Tissier and Martelly)⁸⁷ has been isolated on occasions from war wounds, and we have found it of frequent occurrence in human fecal matter. Weinberg and Seguin recovered *B. bifermentans* only twice in 92 cases of gas gangrene, so it is probable that *B. bifermentans* is more widespread in this country than in France.

At the present time, there seems to be a marked difference of opinion regarding the proteolytic ability of *B. bifermentans*. Tissier and Martelly originally pronounced it a strongly proteolytic species, and Weinberg and Seguin are of the same opinion. Hall claims his strains to be strongly proteolytic, while Kendall, Day and Walker did not seem to find chemical or cultural evidence to that effect. The cultures we isolated from single cells showed proteolysis, but definitely mild in type and entirely incomparable to the proteolytic phenomenon produced by *B. sporogenes* or *B. botulinus*.

B. bifermentans is a large, thick, gram-positive bacillus with spores usually situated centrally. The organism occurs singly, in pairs, and in short chains. The average size is about $4 \times 1.0 \mu$. The spores are rather large and strike one as being oblong rather than oval. There is no swelling at the point of sporulation.

On cooked meat medium, *B. bifermentans* acts in a rather characteristic manner. In some cases, the meat is darkened after 24 hours' incubation, leading one to believe that the organism will subsequently be classed as a strongly proteolytic type. In other cultures, the meat takes on a mottled appearance produced by alternate areas of brownish red and black. Gas is evolved but usually in small amounts. Actual digestion is rather feeble, often not embracing more than one-quarter of the meat. This is variable, however, as strains have

⁸⁴ Jour. Path. & Bacteriol., 1917, 21, p. 334.

⁸⁵ Jour. Path. & Bacteriol., 1918, 22, p. 1.

⁸⁶ Mitt. a. Kaiserl. Gesundheits., 1881, 1, p. 51.

⁸⁷ Ann. de l'Inst. Pasteur, 1902, 16, p. 866.

been isolated from stools which are somewhat more marked in their proteolytic ability. No strains were isolated which were as markedly proteolytic as *B. sporogenes*. Egg albumin cubes are attacked in a rather characteristic way, giving rise to a mucoid, slimy growth product; with the possible exception of a "biting in" at the edges of the cube, there is little other evidence of digestion. The cube of egg albumin usually rests on the mucinous product elaborated, and at first glance one is convinced that the albumin has been broken down, but closer scrutiny will usually reveal that the cube remains unaltered as regards its total volume. Milk is clotted, and on this medium digestion proceeds further than on any of the others. Inspissated serum is darkened, and there is evidence of feeble digestion. Gelatin is darkened and usually liquefied after 48 hours. Indol is elaborated by *B. bifermentans* in suitable sugar-free mediums.

The cultures of *B. bifermentans* isolated by us fermented glucose, levulose, galactose, maltose and glycerol. Lactose was not fermented. As regards the power of this species to ferment glycerol, there seems to be some slight variation in the individual culture. Most of the strains seem able to utilize it, but two cultures have been isolated from stools identical in all other cultural features, which only reduced the H-ion concentration of the medium containing glycerol from P_H 7.2 to 6.7. This feeble reaction may possibly have been due to an unusual amount of a buffer substance present in the medium. Inulin was not attacked by any of the cultures of *B. bifermentans* isolated by us.

Observers all agree that *B. bifermentans* is without pathogenic properties when injected into laboratory animals.

B. bifermentans produces no hemolysins. This species has been isolated from stools on numerous occasions.

B. putrificus (Bienstock).⁸⁸—The identification of *B. putrificus* has been under discussion for a considerable time. In 1906, Bienstock⁸⁹ undertook a redescription of the type, as it was then assumed, and probably correctly, that his original cultures were impure. He subsequently stated that *B. putrificus* was a nonpathogenic, strict anaerobe, having oval spores terminally situated, rapidly digesting coagulated albumin, but having weak saccharolytic properties. This species was not found in the normal intestine by Bienstock, but its presence there was attested to by Passini,⁸ Rodella,⁹⁰ Metchnikoff¹³ and Rettger.¹² Sturges and Rettger⁹¹ found that *B. putrificus* exerted marked proteolytic ability only when grown in association with such aerobes as *Staphylococcus aureus* or *B. coli*. Hall was unable to confirm this. The British Medical Research Committee⁷⁷ believed that *B. putrificus* represented a mixture of *B. tertius*, *B. cochlearius* and *B. sporogenes*. Hall investigated cultures of *B. cochlearius* procured from the English investigators, and found them identical with *B. putrificus*. There is no doubt in his mind that *B. putrificus* is a separate species, or at least an outstanding representative of a closely related group and should be treated as such. Recently, Reddish and Rettger⁹² have reinvestigated their strains and found them to be slowly, but, after 10 days, rather markedly but weakly proteolytic, having, however, no carbohydrate-splitting ability. I have attested to the definite but mild proteolysis prompted by this species⁵³ as well as other cultural features recently reported by Reddish

⁸⁸ Ztschr. f. Klin. Med., 1884, 8, p. 1.

⁸⁹ Ann. de l'Inst. Pasteur, 1906, 20, p. 407.

⁹⁰ Ibid., 1905, 19, p. 804.

⁹¹ Jour. Bacteriol., 1919, 4, p. 171.

⁹² Jour. Bacteriol., 1923, 8, p. 375.

and Rettger, and see no justification in their claim that Metchnikoff, Ashoff, Kendall, Day and Walker, and myself, have been working with a species of anaerobe different from their strain of *B. putrificus*. In our initial description of the cultural features of *B. putrificus*, we attested to the ability of this species to ferment maltose. This characteristic has been found wanting in some of the cultures subsequently isolated from stools but present in others. The strains fermenting this sugar may be closely related to the so-called para-*putrificus* group.

This species seems gram-positive, but many individuals have a decided gram-negative tendency. The spores are oval to round, and usually situated terminally. *B. putrificus* occurs singly, in pairs, and in small groups. In young cultures, no long chain forms are to be noted. In size it usually measures about $2.5 \times 0.3 \mu$. Many forms are to be seen in which the spore stains as well as the bacterial rod. Sporulating types are not numerous in young cultures.

In cooked meat medium, after 24 hours' incubation, the meat is darkened in the upper layers, and a small amount of gas is produced. A foul odor is elaborated, but digestion of the meat is not apparent until after 10 days, when the meat is reduced about one-quarter in volume. (Proteolysis does not proceed further than this for many weeks.) Cubes of egg albumin are at times rendered translucent and may be digested after 10 days in the incubator. Milk is slowly clotted, and finally about one-half of the clot is digested. Inspissated serum is slowly darkened (10 days) and weak digest takes place. Gelatin is darkened and usually liquefied in 3 days. The proteolytic characteristics of *B. putrificus* are quite marked, but digestion is slow and gradual, and the end points reached are not, with the exception of solidified egg albumin and gelatin, as far advanced as those produced by members of the strongly proteolytic group. Glucose, levulose, galactose, saccharose, raffinose, xylose, glycerol and inulin are not attacked. Some strains have been isolated from stools which are identical in other respects to the others, but which feebly ferment maltose. *B. putrificus* elaborates methemoglobin.

This species, as has been repeatedly observed, is not pathogenic for laboratory animals. I have isolated it from stools on several occasions.

B. tetani (Nicolai⁹³).—The cultural features of *B. tetani* have been described differently by a number of investigators. On one feature, however, all of them agree, and that is, the ability of this species to elaborate a powerful and fatal toxin. Early observers attributed to this organism a marked carbohydrate-splitting ability. Achalmé,⁹⁴ in contradiction to this, was among the first to state that *B. tetani* was unable to utilize any carbohydrate. Mace⁹⁵ arrived at the same conclusion as did Adamson and Cutler⁹⁶ some years later. Hall, who for the past several years has been working on *B. tetani*, confirmed these findings, as did Kendall, Day and Walker. Regarding the proteolytic abilities of *B. tetani*, most investigators agree that they are feeble.

The different results obtained by Hall and by Kendall et al. and myself on the ability of *B. tetani* to ferment glucose, saccharose, maltose, lactose, raffinose, mannose and xylose (I reported positive fermentation for these sugars, while the foregoing investigators obtained negative results), led me to retest recently my cultures of *B. tetani* in the presence of these substances, with results more in accord with those obtained by others. Acid formation is not

⁹³ Deutsch. med. Wehnschr., 1884, 10, p. 842.

⁹⁴ Ann. de l'Inst. Pasteur, 1902, 16, p. 663.

⁹⁵ Traité Pratique de Bacteriol., 1913.

⁹⁶ Lancet, 1917, 192, p. 688.

nearly as marked as originally reported by me. At times I have found the P_H will have been reduced from 7.2 to 6.8 or 6.7 in the presence of some carbohydrates, but this is now regarded as being almost a negative, fermentative reaction.

The strains used in my first investigation were all isolated from single cells and on reexamination have been found to be absolutely pure. The mediums previously used had been carefully tested for sterility and sugar-free properties before the tests were made. In spite of these precautions, some untoward factor undoubtedly became manifest and gave me the false conclusions reported. On again going over our data, the only factors I can attribute this mistake to are contamination at time of inoculating or acid in the glassware.

B. tetani does not sporulate profusely, even in sugar-free medium. It is a large, gram-positive organism, but quite a few forms stain with irregularity, giving rise to a peculiar banded or beaded appearance. The spores are round and situated terminally. In size, *B. tetani* average about $3 \times 1 \mu$. The ends are usually pointed or slightly rounded.

B. tetani does not seem to be able to utilize any of the carbohydrates as a nonnitrogenous source of energy.

On cooked meat medium, after a short incubation, the meat is turned a darkish gray, and a small amount of gas is produced. The meat is slowly and feebly proteolyzed, and after about 2 weeks is reduced some 25% in volume. A slight foul odor accompanies this reaction. Coagulated egg albumin is partially digested, but not reduced in volume more than one-half. The disintegrated substance remains as a slimy, stringy halo about the unattacked portion of the cube. Milk is clotted (probably due to a rennin-like ferment), but there is slight, if any, visible digestion of the clot. Inspissated serum is darkened, and a small amount of gas is produced, but there is no clearly defined evidence of actual digestion. Gelatin is darkened and liquefied. *B. tetani* elaborates a powerful hemolysin.

B. tetani produces a potent and fatal neurotoxin which causes lethal effects in white mice when injected in doses as small as 0.001 c.c., or less than that, of culture.

I did not succeed in isolating *B. tetani* from the stools in any of the cases examined. Tenbroeck and Bauer,⁹⁷ Peking, recovered this species from 34% of 79 fecal specimens examined, all from normal persons. They have since been able to demonstrate⁹⁸ tetanus antitoxin in the blood of a large majority of the patients showing the presence of this organism in the stool, while in cases in which *B. tetani* was not found, the blood was almost uniformly negative for the presence of the specific antitoxin.

The widespread infection of the Chinese about Peking with *B. tetani*, in contradistinction to our results regarding the presence of this species in the stools of persons living in New York City, may possibly be interpreted as follows. The use of night soil as a fertilizer is widespread among the market gardeners about Peking and very limited about New York City. Thus, the medium of infection would be more general in China.

B. tetanomorphus (*B. pseudotetani*) (Sanfelice).⁹⁹—Morphologically, this species differs slightly from *B. tetani* in that the spores of *B. tetanomorphus* are slightly more oval than round in some of the forms, while others have the spore subterminally situated.

⁹⁷ Jour. Exper. Med., 1922, 36, p. 261.

⁹⁸ Ibid., 1923, 37, p. 479.

⁹⁹ Ztschr. f. Hyg. u. Infektionskrankh., 1893, 14, p. 339.

The difference in carbohydrate reaction between *B. tetanus* and *B. tetanomorphus* is that *B. tetanomorphus* ferments glucose. As regards proteolytic activities, *B. tetanomorphus* does not digest or darken cooked meat medium; it does not digest solidified egg albumin, inspissated serum, nor alter milk. Gelatin is liquefied but not darkened. No toxin-producing or pathogenic characteristics have been noted for this species. *B. tetanomorphus* has been isolated from one stool specimen in the present series. As far as I know, there is no difference of opinion concerning the identity of this type.

B. aerofœtidis (Weinberg and Seguin).¹⁰⁰—This interesting species was first isolated by the French observers from infected war wounds. Henry¹⁰¹ confirmed their observations on the occurrence of this species and did considerable work on its reactions in the presence of various carbohydrates, extending somewhat the list of these substances fermented as initially reported by Weinberg and Seguin. My results have been shown to agree, for the most part, with those reported by Henry.

One of the features characterizing this species is the infrequent occurrence of sporulating types even when the organism is grown in a medium especially designed to induce sporulation. In confirmation of Bullock's¹⁰² observations, I also have found the most common position of the spore to be subterminal.

As far as I know, there is no disagreement among bacteriologists concerning the cultural features of this species. It is a short, plump, gram-positive bacillus with rounded ends, and occurs singly and in pairs. The average size is about $1.8 \times 0.6 \mu$. The spores are small, round to oval, and situated subterminally.

B. aerofœtidis is feebly proteolytic. On cooked meat medium, there is darkening of the meat, some production of gas, and a slight foul odor. Prolonged incubation, however, shows only a moderate reduction in the total volume of the meat. Milk is clotted and darkened, but not visibly digested. Solidified cubes of egg albumin are feebly attacked about the margins. Inspissated serum is darkened and somewhat more energetically digested than the other substances designed to show proteolysis. Gelatin is darkened and slowly liquefied.

Glucose, levulose, galactose, lactose, maltose, mannose and xylose are actively fermented with acid and gas production. Salicin is feebly attacked, but I do not think it should be taken as a constant cultural feature as some of the strains isolated did not visibly utilize it. Saccharose is not fermented nor is inulin or glycerol. A positive lactose reaction and a negative saccharose reaction serve, on the basis of carbohydrate fermentation, to differentiate this species from others resembling it.

B. aerofœtidis is not hemolytic.

Weinberg and Seguin consider *B. aerofœtidis* to be weakly pathogenic. This species has been isolated on occasion from the cases studied.

B. welchii (Welch and Nuttal).¹⁰³—*B. welchii* has been known under a variety of names. Among the most outstanding of these are "*B. aerogenes capsulatus*," "the gas bacillus" and "*B. perfringens*." The latter term is still widely used in Europe. The term "*B. welchii*" seems to have been universally accepted by American observers.

Prior to recent times the cultural features of this species or group were the subject of much controversy. Older workers, among whom may be mentioned Klein, Tissier and Martelly, Distaso, Herter, and Rettger, attributed

¹⁰⁰ Compt. rend. Soc. de biol., 1916, 79, p. 116.

¹⁰¹ Jour. Path. & Bacteriol., 1916, 21, p. 367.

¹⁰² Ibid., p. 344.

¹⁰³ Bull. Johns Hopkins Hosp., 1892, 3, p. 81.

marked proteolytic ability, as well as other foreign cultural features, to *B. welchii*. It is beyond the scope of this study to quote the large volume of literature which has grown up about this species. Suffice it to say that it is obvious that the cultures described by these early workers either contained other types in addition to *B. welchii*, or that the species we now recognize as such was not present at all. The admirable reports of Simonds³² and Jablons¹⁰⁴ have done much to clarify the cultural features of this group in the light of present-day methods. We are now able, with the aid of characteristic reactions, to recognize *B. welchii* with comparative ease.

On the basis of the ability of some strains of *B. welchii* to ferment inulin or glycerol, in addition to the other carbohydrates, Simonds has divided the species into 4 types: Type 1 ferments glycerol and inulin; type 2 ferments glycerol but not inulin; type 3 ferments inulin but not glycerol; type 4 ferments neither inulin nor glycerol.

This method of grouping has been adopted throughout the present investigation, but I have found on occasion a number of intermediate forms showing a gradation from one type into another. For instance, in some type 4 strains there may be a slight tendency to ferment either inulin or glycerol, but in these cases the reaction is not nearly as clearly defined as in a frank type 2 or type 3 strain.

In spite of the large amount of work done on the *B. welchii* group, there still exists some difference of opinion concerning a few of the less important cultural features. These will be discussed as they arise.

B. welchii is morphologically rather characteristic. It is strongly gram-positive, occurs singly, in pairs, and in short chains. It is relatively large, averaging in size about $5 \times 0.7 \mu$. The ends are typically square, but in some forms may be slightly rounded. *B. welchii* does not sporulate in mediums containing fermentable carbohydrate. In sugar-free medium, sporulating types usually have the spore situated centrally or slightly subterminally. Hall and numerous other observers have never found spores of *B. welchii* in infected living animal tissue, although cultural tests confirm the formation of spores in the human intestine.

Most of the commonly used carbohydrates are fermented by *B. welchii* with the elaboration of large amounts of acid and gas. Arabinose, salicin and mannose are not visibly attacked, although Hall found one strain which did utilize salicin. This species varies in its ability to ferment inulin, glycerol or both, according to the type, as described above.

The differences of opinion in regard to the cultural features of *B. welchii* are mostly focused on its ability to digest protein, and as regards this, I steadfastly hold to my views already published, that there is no visible evidence of such activity on mediums designed to show proteolysis. As has already been stated, many of the older observers claimed proteolysis as a cultural feature of *B. welchii*. Shattenfroh and Grassberger were among the first to call attention to the fact that such is not the case. Hall states that "Welch cultures are only mildly proteolytic. Brain medium is neither blackened nor liquefied, but is slightly softened." Wolf and Harris¹⁰⁵ investigated the metabolic products of *B. welchii* and found action on protein and protein derivatives to be minimal even in the absence of utilizable carbohydrates. Kendall, Day and Walker support this view, which is in accord with that of Simonds.

¹⁰⁴ Jour. Lab. & Clin. Med., 1920, 5, p. 374.

¹⁰⁵ Jour. Path. & Bacteriol., 1917, 21, p. 386.

On cooked meat medium, *B. welchii* gives a characteristic reaction. Gas is elaborated in large amounts, and the meat is turned a deep red-carmin when the medium is fresh and properly made. None of the strains studied by me produced black pigmentation on this medium. Although the individual particles of the meat were somewhat reduced in size, I do not believe that there occurs actual digestion. There is little or no diminution of the total volume nor other evidence of proteolysis of this substance, even after prolonged incubation. Milk is attacked with the characteristic "stormy fermentation" which is so well known as to need no further description here. Solidified egg albumin cubes and inspissated serum are not digested. Gelatin is constantly liquefied. This fact is also attested to by Hall and others. Kendall and his co-workers report a marked "softening" of gelatin, but were unable to demonstrate a gelatinolytic ferment. *B. welchii* is markedly hemolytic.

B. welchii seems to vary as regards its pathogenic properties. In Hall's recent contribution, he has divided the strains into the following: non-pathogenic, moderately pathogenic and strongly pathogenic. In testing for virulence with guinea-pigs, he found that when sublethal doses of the strongly pathogenic types were given, the animals frequently suffered a profound intoxication associated with the characteristic gaseous edema. When the acute infection subsided, the skin usually sloughed from the site, leaving an open wound of such magnitude as frequently to cover the thorax and abdomen. These ulcers tended to heal, leaving a clean white scar. In the test animals used, only one died, and this was found to have been due to a hernia arising from accidental perforation of the abdominal wall. Recovered animals developed only a slight immunity either to reinfection with the same strain or infection with others.

Bull and Pritchett¹⁰⁶ have reported that in cultures of *B. welchii* of 48 hours or older, there is a marked decrease of the toxic principles elaborated. This observation has been confirmed by the Kendall group, Hall and others. The presence of fermentable carbohydrates seems to have such an influence in reducing the production of the poison that the striking decrease in potency after 48 hours has been thought to be due to neutralization of the toxic principles by products incidental to growth or by the extreme lability of the substance. The former seems to be the case, as it has been shown that filtrates rendered sterile show no decrease in potency even after standing, showing no lability in the actual sense of the word.

B. welchii has been isolated from a large majority of the cases here studied.

Vibrio septique (Pasteur)¹⁰⁷.—The long standing confusion concerning the identity of the bacillus of malignant oedema Koch, and Pasteur's organism may now be said to have cleared. The efforts of Meyer,¹⁰⁸ Weinberg and Seguin and Heller¹⁰⁹ have done much to untangle this complicated situation, and it now seems established that Koch's cultures were composed principally of *B. sporogenes*, and the highly putrefactive action reported by him was due to the presence of this species, which either overgrew the pathogenic form or was the only organism isolated.

Vibrio septique is a highly toxic anaerobic spore-bearer which has been found on numerous occasions in gangrenous war wounds. Culturally, it is slightly like *B. welchii*. It is classified by me as strictly a nonproteolytic type.

¹⁰⁶ Jour. Exper. Med., 1917, 26, p. 119.

¹⁰⁷ Bull. de l'Acad. de méd., 1877, p. 783.

¹⁰⁸ Jour. Infect. Dis., 1915, 17, p. 458.

¹⁰⁹ Ibid., 1920, 27, p. 385.

Vibrio septique seems to be possessed of slight pleomorphism. It is gram-positive, but does not in all instances retain the gentian violet very definitely. On sugar-free mediums forms with terminal spores occur frequently, as is also the case in mediums containing fermentable carbohydrates. Smears taken from animals inoculated with this species show a wide variation in morphology, varying from long filaments to much shorter, thicker bacilli. This characteristic was reported by Hall and by Weinberg and Seguin. On sugar-free medium, the average size may be taken as $2.5 \times 0.3 \mu$. The spores are usually large and oval.

On cooked meat medium, gas is evolved in considerable amounts, and the meat is turned to a bright copper red, but not as deep red as that produced by *B. welchii*, nor is there usually as much gas produced. There is no evidence of digestion of the meat fibers even on prolonged incubation. Milk is slowly clotted with small evolution of gas, but there is no subsequent alteration in the clot. Inspissated serum and solidified egg albumin are not attacked. Gelatin seems to be liquefied by all strains.

My studies show that *Vibrio septique* ferments glucose, levulose, galactose, saccharose, maltose, lactose and salicin. Inulin and glycerol are not attacked. The strains studied by Hall seemed void of saccharose-fermenting ability, while those reported by Kendall, Day and Walker possessed this property in agreement with my results. *Vibrio septique* produces hemolytic substances; this serves partially to differentiate it from *B. oedematis*, a more or less closely related form.

This species elaborates a potent toxin similar in most respects to that produced by *B. welchii*. It is most powerful during the early hours of growth. Kendall, Day and Walker have found nothing in the metabolic products of this species to account for its extreme toxicity. When cultures of *Vibrio septique* are contaminated with other bacteria, the strength of the poison seems to be rendered nil. Subcutaneous injections of young cultures of *Vibrio septique* produce external lesions similar to those of *B. welchii*. When intramuscular injections were made with my cultures, the guinea-pigs appeared to be very intoxicated and died on the second day. The differences apparent in the lesions of animals dead of *Vibrio septique* or *B. welchii* infection have been reported in great detail by Weinberg and Seguin, Robertson,¹¹⁰ and the British Committee, so that no further space will be given to them here. Smears taken from the peritoneum of infected animals frequently show long granular filaments, supposed by many to be of considerable diagnostic value. This species I have isolated from stools on two occasions.

B. oedematis (Weinberg and Seguin¹¹¹).—This species, which is closely related to *Vibrio septique*, was isolated at the beginning of the war from a number of infected wounds. It is probably identical with, or very similar to, *B. oedematis maligni* II of Novy, and accordingly, Hall, considering the name "*B. novyi*" to have priority, has so designated the cultures of this species on which he reported. As Novy named the organism "*B. oedematis maligni* II," which is not such a far cry to the term "*B. oedematis*," the latter meaning much more regarding the characteristics of the species than the name of its original observer, it would seem proper that the name "*B. oedematis*" be allowed to stand. Kendall and his co-workers also used this name, as did the French observers.

A subterminally sporulating bacillus staining gram-positive, but at times rather irregularly so, many forms giving a mottled or beaded appearance. It is

¹¹⁰ Brit. Med. Jour., 1918, 1, p. 585.

¹¹¹ Compt. rend. Soc. de biol., 1915, 78, p. 507.

variable in size, however. A number of forms measure approximately 2.5 or 3x1.0 μ . *B. oedematiens* occur singly, in pairs and in small groups. No chain forms were noted. The spores are oval and the ends rounded.

B. oedematiens is frankly a member of the nonproteolytic group. On cooked meat medium, there is an elaboration of considerable gas; the meat is colored a light red, which gradually fades to salmon pink, but even after prolonged incubation there is no evidence of digestion. Milk is clotted after 4 days, but there is no further action. Coagulated egg albumin and inspissated serum are not attacked. There is no visible evidence of proteolysis on any of the mediums. Gelatin is liquefied after 3 or 4 days.

Regarding the carbohydrophilic propensities of this species, there is considerable difference of opinion. In repeated tests, our cultures of *B. oedematiens* have been found to act as follows. Glucose, levulose, galactose, saccharose, maltose, lactose, raffinose, mannose and starch are regularly fermented. Inulin and xylose are at times feebly attacked, but should not be taken as a constant feature. Glycerol is not utilized. *B. oedematiens* differs from *Vibrio septique* in that it does not ferment salicin but does attack starch. The results obtained by Henry agree for the most part with mine. Wolf¹¹² claimed that his strains did not ferment galactose, saccharose or lactose. Hall obtained positive reactions with glucose and glycerol, but negative results with lactose, saccharose, salicin and inulin.

A point of value in separating *B. oedematiens* from *Vibrio septique* is that *B. oedematiens* is nonhemolytic while *Vibrio septique* is hemolytic.

There seems to be no question that *B. oedematiens* is a highly pathogenic organism, although Weinberg and Seguin, and also Hall, have demonstrated that some strains are less virulent than others. Cultures isolated by me from human feces invariably produced massive edema and death in 48 hours when injected intramuscularly into guinea-pigs. The edema is somewhat less gaseous than that produced by *B. welchii* or *Vibrio septique*. Kendall, Day and Walker and I have isolated this species from human stools.

B. fallax (Weinberg and Seguin¹¹³).—Primarily isolated directly from infected wounds, *B. fallax* has also been obtained from the blood stream during the early stages of wound infection.

B. fallax is a small, slender bacillus with gram-positive staining properties. As a more or less common occurrence with representatives of the nonproteolytic group, some of the individuals retain the violet dye but lightly. Few spores or spore-bearing types occur in mediums containing fermentable carbohydrates. In sugar-free mediums the spores, which are oval, are usually situated terminally or slightly subterminally. Nonspore-bearing forms are usually very thin, giving rather a characteristic appearance of tapering toward each end. The organism measures about 2x0.2 or 0.3 μ .

In cooked meat medium, no growth is usually apparent until after 48 hours. Gas is at that time sparsely formed, and the meat assumes a light red color which fades to salmon pink after the third day. In my strains there was no production of black pigment on this medium as reported by Henry. There is no visible alteration in the volume of the meat. Milk is slowly clotted, usually not until after two weeks of incubation. It is then formed into a loosely organized mass, and there is no further evidence of activity. Solidified egg albumin and inspissated

¹¹² Jour. Path. & Bacteriol., 1920, 23, p. 254.

¹¹³ Compt. rend. Soc. de biol., 1915, 78, p. 686.

serum are not altered in any way. Gelatin is not liquified by any of the strains which I studied. This is in harmony with the observation of Kendall et al.

In my hands, cultures of this species fermented glucose, levulose, galactose, saccharose, maltose, lactose and xylose. Mannose was feebly attacked by some cultures and not at all by others. Positive results were not obtained for glycerol, inulin or salacin. Henry reports that lactose is not utilized, while the results of Kendall, Day and Walker agree with mine. The latter observers also report negative results with glycerol for some of their strains but slightly positive for others. They seem somewhat dubious about this point, stating that the possibility of the glycerol used by them being impure was not altogether remote. Methemoglobin is produced.

The pathogenic properties of this species have been studied by Weinberg and Seguin and by Henry. They agree that recently isolated cultures of *B. fallax* are toxic for guinea-pigs but that the organism loses this property on artificial cultivation. When injected intramuscularly into rabbits or guinea-pigs, it produces a marked gaseous edema with reddening of the muscles. When injected intravenously into rabbits in doses of 1 c.c. death usually occurs in from 12 to 16 hours.

The following features are sufficient to differentiate *B. fallax* from other closely related species: nonliquefaction of gelatin, milk very slowly clotted (10-20 days), mannose feebly attacked, xylose energetically fermented; characteristic morphology; elaboration of methemoglobin.

B. tertius (Henry¹¹⁴).—Henry so named this species because it was found by him to be the third most frequent of the anaerobic spore-bearers in occurrence in war wounds.

Sporulating forms of *B. tertius* are frequently seen even in mediums containing fermentable carbohydrates. It is a relatively long, slender bacillus and gram-positive. Some of the forms do not stain deeply. The spore-bearing types seem, on the whole, somewhat longer than the vegetative forms. The spores are oval or roundish and usually situated terminally. Immature spores possess the faculty of retaining at times the violet dye. In size, *B. tertius* measures about $4 \times 0.4 \mu$. No chain forms were noted.

On cooked meat medium, a large amount of gas is formed, somewhat resembling *B. welchii* in intensity. The meat is reddened but soon fades to a light copper color. There is no proteolysis or production of black pigment. Milk is clotted after 48 hours, and a few bubbles of gas are produced. There is no visible digestion of the clot. Coagulated egg albumin and inspissated serum are not attacked. Gelatin is not liquefied until after prolonged incubation (30-40 days); finally however, gelatin is definitely liquefied.

B. tertius is a frank carbohydrophile. Large amounts of gas are produced when the sugars in question are fermented, but the acid production seems to be somewhat less than that elaborated by other members of the nonproteolytic group under similar conditions. Glucose, levulose and xylose are, on the whole, more energetically attacked than are galactose, maltose and lactose. Saccharose is feebly utilized by some strains, but more markedly so by others. Inulin is feebly attacked by some strains. Salicin and glycerol are not utilized according to my experience. Hall found salicin fermented, but not inulin or glycerol. Hemolytic substances are elaborated by *B. tertius*.

Observers are unanimous in the opinion that *B. tertius* is not pathogenic. I have isolated this species from human feces, as have Kendall, Day and Walker.

¹¹⁴ Jour. Path. & Bacteriol., 1917, 21, p. 344.

B. sphenoides (Douglas, Flemming and Colebrook¹¹⁶).—My observation of this species has been limited to one culture kindly sent to me by Dr. Hall, who describes its cultural and morphologic characteristics as follows:

This is a small, motile, gram-positive rod, fusiform in the vegetative stage. Spores are at first subterminal but become clearly terminal as they mature and are almost round. The opposite end of the rod maintains its pointed form. Spores are set free in great abundance.

No blackening or digestion occurs in brain medium. Blackening and gas production take place in gelatin, but there seems to be no liquefaction. Considerable gas is evolved from milk, but clotting does not take place. Indol is not formed.

Glucose, lactose and salicin are fermented, with acid and gas production. Saccharose, inulin and glycerol were not fermented by the strains studied by Hall.

Hall has not been able to demonstrate pathogenic characteristics through subcutaneous or intravenous inoculations into guinea-pigs and rabbits.

Anaerobic bacteria, resembling *B. sphenoides* culturally or morphologically, have not been isolated by me from human stools.

B. chauvœi (Kerry¹¹⁶).—As has been shown by Heller,¹⁰⁹ this species, together with *Vibrio septique* and *B. oedematiens*, is responsible for black-leg infection in cattle. As far as we know, *B. chauvœi* has not been isolated from human sources. It has been pointed out that, on the other hand, and it is interesting to note, *B. welchii* has not been associated with black-leg infection.

B. chauvœi is a relatively medium-sized gram-positive bacillus, freely forming subterminal spores. At times the spores stain as well as the bacterial cell. It occurs singly, in small groups and pairs, but no chain forms have been noted. In size *B. chauvœi* measures about $3 \times 0.4 \mu$.

In cooked meat medium, there is considerable evolution of gas. The meat assumes a pale copper hue, but is not digested in the least. The strain studied by me clotted milk after 6 days' incubation, converting the substance into a loosely organized mass. Solidified egg albumin and inspissated serum are attacked. Gelatin is liquefied after 4 days.

Glucose, levulose, galactose, maltose and lactose are fermented with acid and gas production. Salicin, inulin and glycerol are not altered. These results correspond with those of Hall and Robertson.

B. chauvœi seems to be highly toxic for guinea-pigs and much less so for rabbits.

I have not been able to isolate this form from human intestinal sources.

B. butyricus (Adamson¹¹⁷).—This species is probably identical with Gruber's *B. amylobacter* and the Buttersaure bacillus of Shattenfroh and Grassberger. It is described by the Medical Research Committee as being a nonliquefier of gelatin. A single culture sent to me by Hall liquefied gelatin in 6 days. Hall reported a similar observation with one of his strains.

B. butyricus is a thin, gram-positive bacillus forming central or subterminal spores sparsely. Many individuals do not retain the violet dye tenaciously. It occurs singly and in pairs; no chain forms were noted. The ends are round or pointed. *B. butyricus* measures some $3 \times 0.3 \mu$.

Gas is elaborated in considerable amount on cooked meat medium, and the meat is turned a dark red, similar to the action of *B. welchii* on this medium.

¹¹⁶ Committee on Anaerobic Bacteria, 1919, Series 39.

¹¹⁶ *Centralbl. f. Bakteriol.*, 1894, 16, p. 372.

¹¹⁷ *Jour. Path. & Bacteriol.*, 1919, 22, p. 345.

Digestion does not take place. Milk is loosely clotted, but there is no alteration of the clot. Coagulated egg albumin and inspissated serum are not digested. Gelatin was liquefied in 6 days.

The fermentative characteristics of this species have not been studied by me. Hall finds glucose, lactose, saccharose and salicin fermented, while glycerol and inulin remain unaltered.

No pathogenic features have been demonstrated for this species.

Anaerobes of this type, closely resembling *B. welchii*, but differing in their reaction on milk, gelatin and as concerns morphology and pathogenic features, were not isolated by me during this investigation.

PRESENTATION OF CASES

With the aid of the cultural information and technic just described, I have isolated and grouped the anaerobic bacteria found in the feces of some 64 persons who presented symptoms of one kind or another which led the attending physicians to believe that a determination of the bacterial types found in the stool might possibly throw some light on the cause of the conditions presented, which, for the most part, were of obscure origin. Examinations were also made of the stools of 10 normal persons who functioned as controls. In this investigation, attention has been paid only to the determination of the anaerobic spore-bearing types and what, if any, information could be gleaned from their presence.

It will become apparent that the number of cases studied is not large and that there is a certain hazard connected with attempting to draw definite conclusions from these comparatively small groups. This, in a measure, is quite true, but, on the other hand, it is difficult to collect a large series of cases all having similar symptomatology, especially as regards the obscure conditions so often associated with intestinal putrefaction and fermentation. I feel that these cases are, to a certain degree, typical, and I have endeavored to group together only such cases as presented closely allied conditions. Surveying the anaerobic bacteria of a single case is a more time-consuming undertaking than most bacteriologic analyses. Throughout this investigation the aim has been to make the survey of each case complete and to identify correctly each organism isolated. Is it not better to gain a thorough knowledge of a limited number of cases than the more superficial understanding that the examination of a very large series would necessarily involve?

The cases studied have been separated into 5 different groups: (a) Intestinal toxemia, (b) chronic diarrhea, (c) chronic eczema, urticaria, skin affections, (d) chronic arthritis, (e) pernicious anemia.

In order to conserve space, a detailed account of each case will not be given but rather one or two of the most typical ones from each group will be outlined for the sake of clarity. A more detailed account of the findings will be found in the discussion and also in the tables in which separate value has been given to incidence of species, numerical estimation and the proteolytic potentialities of the combined anaerobic spore-bearing flora on cooked meat medium.

Intestinal Toxemia, 18 Cases.

Case 1a, a woman, aged 48. Chronic headaches for 16 years; indigestion with flatulence; headaches seemed to be of intestinal origin; no other known cause.

Stool semifluid; dark green; odor not pronounced; much mucus present.

Combined anaerobic spore-bearing flora nonproteolytic on cooked meat medium; typical *B. welchii* reaction; dark red coloration with large amounts of gas.

Anaerobic bacteria numerous; *B. sporogenes* in majority; *B. putrificus*

Case 18a, a man, aged 29. Chronic severe headaches almost daily; marked constipation; nausea at times; general malaise; constipation less marked and symptoms less severe when on low protein diet.

Stool well formed and moist; dark brown; no excess of mucus; odor not especially foul.

Combined anaerobic spore-bearing flora strongly proteolytic on cooked meat medium.

Anaerobic bacteria numerous; *B. sporogenes* in majority; *B. putrificus*. *B. welchii*, type 3.

Chronic Diarrhea, 6 Cases.

Case 1b, a woman, aged 55. Frequent attacks of diarrhea; nervous and excitable; fatigue, backaches and headaches.

Stool fluid; odor rather foul; brown; mucus not in excess.

Combined anaerobic spore-bearing flora nonproteolytic on cooked meat medium; typical *B. welchii* reaction.

Anaerobic bacteria numerous; *B. welchii*, type 4 predominating; *B. sporogenes*.

Case 6b, a woman, age not given. Persistent diarrhea; pains in the rectum, back and legs.

Stool fluid; odor rather foul; light brown; some excess of mucus.

Combined anaerobic spore-bearing flora feebly proteolytic on cooked meat medium.

Anaerobic bacteria few; *B. bifermentans*, predominating; *B. fallax*; *B. welchii*, type 2.

Chronic Eczema and Urticaria, 8 Cases.

Case 1c, a woman, aged 27. Chronic eczema on face and hands; indigestion; malaise.

Stool well formed; dark brown; odor not especially foul; mucus not in excess.

Combined anaerobic spore-bearing flora strongly proteolytic on cooked meat medium.

Anaerobic bacteria numerous; *B. centrosporogenes*, in majority; *B. putrificus*; *B. welchii*, type 4.

Case 7c, a man, aged 27. Marked urticarial eruption; itching of the skin of face; at times swelling and numbness of legs.

Stool fluid; greenish brown; odor not especially foul; no excess of mucus. Combined anaerobic spore-bearing flora nonproteolytic on cooked meat medium.

Anaerobic bacteria numerous; *B. welchii*, type 4, in large majority; *B. tertius*; *B. sporogenes*.

Chronic Arthritis, 6 Cases.

Case 2d, a man, aged about 45. Subject to acute flare-up of pains in joints; both knees and wrists badly involved. Tonsils out; teeth normal; no respiratory or genito-urinary infection; all other foci of infection ruled out.

Stool well formed and moist; odor not foul; light brown; no excess of mucus.

Combined anaerobic spore-bearing flora nonproteolytic on cooked meat medium although few darkened meat particles.

Anaerobic bacteria few; *B. welchii*, type 4, in majority; *B. bifermentans*.

Case 4d, a woman, aged 50. Stiffness of neck; pains in shoulders; headaches; exhaustion; nervousness.

Stool semiformed; greenish brown; no excess of mucus; odor foul.

Combined anaerobic spore-bearing flora nonproteolytic on cooked meat medium.

Anaerobic bacteria numerous; *B. sporogenes* in large majority; *B. tertius*; *B. welchii*, type 2.

PERNICIOUS ANEMIA, 24 CASES

Studies of the anaerobic spore-bearing flora of the pernicious anemia cases have been made in conjunction with Dr. L. Mary Moench of the Mayo Foundation, Rochester, Minn. The details of this investigation will shortly be reported on separately. These cases were, on examination, found to be so uniform in regard to the species of anaerobic spore-bearing bacteria isolated that it was thought feasible to list the results in tabular form. Accordingly, they will be found summarized in table 1.

TABLE 1
PERNICIOUS ANEMIA CASES

Case No.	Action of Combined Anaerobic Flora on Cooked Meat Medium	Predominating Anaerobe
4, 5, 6, 8, 11, 12, 14, 18, 19, 20, 21, 22	Nonproteolytic; typical <i>B. welchii</i>	<i>B. welchii</i> , type 1
15, 16,	Nonproteolytic; typical <i>B. welchii</i>	<i>B. welchii</i> , type 2
10, 17, 23, 24,	Nonproteolytic; typical <i>B. welchii</i>	<i>B. welchii</i> , type 3
1, 2, 3, 7, 9, 13,	Nonproteolytic; typical <i>B. welchii</i>	<i>B. welchii</i> , type 4

It will be seen that out of the 25 cases examined, *B. welchii* was overwhelmingly numerically superior to all other species, in most of the cases entirely masking their presence. *B. sporogenes* has been isolated from the feces of 2 of these cases, but in numbers so small in comparison to the *B. welchii* count, that their presence was taken to be entirely insignificant.

Of the 25 cases examined, it will be seen that type 1, *B. welchii* was present 12 times (50%); type 2, twice (8%); type 3, 4 times (16%);

and type 4, 6 times (25%). Of the cases comprising this series, it may be said that they were all diagnosed as pernicious anemia, giving the typical blood picture and other characteristic symptoms. Several of the patients have since died of the disease. It is important and interesting to note that the *B. welchii* content in the stools in the pernicious anemia cases was uniformly much higher than encountered in the fecal specimens taken from those having the other maladies here under discussion.

ANAEROBIC SPORE-BEARING BACTERIA IN THE FECES OF NORMAL PERSONS
CONTROL CASES, 10

In selecting persons to serve as controls, especial effort was made to obtain persons who, as far as was known, were normal, especially as concerned their digestive activities. Each prospective control case was carefully questioned with the point in view to detect any untoward symptoms, such as headaches, occasional or chronic constipation, flatulus, chronic diarrhea, arthritis, urticaria, eczema and, in general, dietary peculiarities. The persons here serving as control cases were entirely normal in these respects and utilized a general mixed diet. Those known to have marked dietary peculiarities, such as the consumption of a large amount of meat, were also ruled out. Finally 10 persons answering our requirements were selected. It may be added that they were not hospital patients in any sense of the word but all active persons, varying in occupation and going daily about their tasks.

For the sake of conserving space, these controls will not be listed separately. A summary of the results will be found in the tables and in the general discussion.

DISCUSSION AND ANALYSIS OF RESULTS

In discussing the possible relationship of anaerobic spore-bearing bacteria in the intestinal flora to the pathologic conditions represented by the series of cases here examined, several considerations must be constantly kept in mind.

The mere constant occurrence of representatives of this anaerobic bacterial group in the fecal specimens cannot be considered as having any direct bearing on the subject in question, as it is quite possible that spores of these types may be ingested with food in small numbers and thus gain access to the large intestine. On the other hand, the relative importance of the occurrence of large or small numbers of these bacteria in a specimen should not be minimized, for it is not likely that when spore-bearing anaerobes occur in limited numbers, any significance can be attached to their presence.

While, as has often been stated, it is a far cry from test tube to intestine, until suitable experimental methods are devised to reproduce even approximately the complex conditions of the human digestive tube,

we must needs derive what information possible from the comparatively unsatisfactory methods at our disposal. These at least permit, with a technic suitable to the problem, the isolation and identification of the species of anaerobes present, determination of their relationship to other elements of the flora and an accurate analysis in vitro, at least, of their pathologic and biologic characteristics.

The possible symbiotic action of several species of anaerobes present in a given case should also be taken into consideration, as well as the possible interrelation of the anaerobic and the aerobic flora, or the action of the aerobic flora alone, each or all of which may combine to play a part in the production of these and other clinical conditions.

Such problems as individual susceptibility of the host to substances elaborated by these bacteria may also possibly enter in. It is quite conceivable that this may vary widely. Some intestines may be more permeable to these products than others. The variation in virulence between strains of bacteria of the same species may be of importance too. The complexities of diet, no doubt, have a bearing. These factors all combine to make the problem a complex one indeed.

In the present study I have had to be largely content with the isolation and accurate identification of such spore-bearing anaerobes as might be associated with these various clinical conditions, and thus lay the foundation for future work.

Intestinal Toxemia, Series A.—In view of the claims held by many investigators and clinicians that certain types of strongly putrefactive anaerobes are directly concerned in producing the manifestations of this somewhat ill-defined condition, it is interesting to analyze the results obtained from the study of the 18 cases in this series.

From reviewing the data summarized in table 2, it becomes apparent that *B. sporogenes*, the only strongly proteolytic species isolated, is present in 10 out of the 18 cases, or 55%. In the control cases, I find *B. sporogenes* present on 5 occasions, or 50%. From casual observation, this would lead one to believe that no correlation could be attempted between this anaerobe and the disease in question, as this species seems to be present with almost identical frequency in both control and pathologic cases. Tables 3 and 4, however, show how hazardous it is to venture an opinion from results based entirely on the mere occurrence of a type.

These tables show *B. sporogenes* entirely dominating the anaerobic flora in the intestinal toxemia group in 5 cases, or 27% of the total, and

TABLE 2
RELATIVE INCIDENCE OF SPECIES OF ANAEROBIC BACTERIA IN CASES STUDIED

Cases	B. sporogenes		B. centro-sporogenes		B. bifermentans		B. putrificus		B. tetanomorphus		B. aerofaecidis		B. welchii		B. tertius		B. fallax		B. oedematiens		Vibrio septique	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Intestinal toxemia.....	10	55.5	1	5.5	3	16.6	5	27.7	1	5.5	0	0	15	88.8	6	33.3	1	5.5	1	5.5	1	5.5
Chronic diarrhea.....	4	66.6	0	0	2	33.3	1	16.5	0	0	0	0	5	83.3	1	16.5	1	16.5	1	16.5	0	0
Chronic eczema, etc.	3	37.7	1	12.5	1	12.5	3	37.7	0	0	2	25.0	6	75.0	2	25.0	0	0	0	0	1	12.5
Chronic arthritis.....	3	50.0	0	0	1	16.5	1	16.5	0	0	0	0	6	100.0	3	50.0	1	16.5	0	0	0	0
Periculous anemia.....	2	8.0	0	0	0	0	0	0	0	0	0	0	24	100.0	0	0	0	0	0	0	0	0
Controls.....	5	50.0	0	0	3	30.0	3	30.0	1	10.0	2	20.0	9	90.0	3	30.0	1	10.0	0	0	0	0

INCIDENCE OF ANAEROBIC SPORE-BEARING BACTERIA IN CASES EXAMINED

Cases	B. sporogenes	B. centro- sporigenes	B. bifementans	B. putrificus	B. tetano- morphus	B. aerotoetidis	B. welchii 1	B. welchii 2	B. welchii 3	B. welchii 4	B. tertius	B. fallax	B. oedematiens	Vibrio septique	Numerical Index	Proteo- lytic Action of Combined Anaerobic Flora
Intestinal Toxemia																
Case 1 A.....	+	P++	P+++	Few	None
2 A.....	P++	Few	Strongly
3 A.....	+	P++	Numerous	None
4 A.....	+	Few	None
5 A.....	P++++	+	+	Feebly
6 A.....	Numerous	None
7 A.....	P+++	+	Numerous	Feebly
8 A.....	+	P++	+	Numerous	None
9 A.....	+	Few	None
10 A.....	Numerous	None
11 A.....	P++++	+	P+++	Numerous	Strongly
12 A.....	P+++	+	P+++	Numerous	Strongly
13 A.....	+	Numerous	None
14 A.....	P++	+	Few	Feebly
15 A.....	P++	Numerous	None
16 A.....	P++	Few	Strongly
17 A.....	..	+	+	P++	Few	None to
18 A.....	P+++	+	+	Numerous	Strongly
Chronic Diarrhea																
Case 1 B.....	+	P+++	Very nu- merous	None
2 B.....	P+++	+	Numerous	Strongly
3 B.....	+	..	+	P++	Few	None to
4 B.....	P+	P+	+	Very few	Feebly
5 B.....	+	+	+	Very few	None
6 B.....	P++	+	+	Few	Feebly
Chronic Eczema and Urticaria																
Case 1 C.....	..	P+++	..	P+	+	Numerous	Strongly
2 C.....	P++	P+	Very few	Feebly
3 C.....	Few	Strongly
4 C.....	+	+	P+++	Very few	None
5 C.....	+	Numerous	None
6 C.....	+	+	Few	Feebly
7 C.....	+	+	Numerous	None
8 C.....	+	..	P+	Very few	Very feebly
Chronic Arthritis																
Case 1 D.....	P+++	..	+	+	..	P++	++	Numerous	Feebly
2 D.....	+	P+++	Few	None
3 D.....	++	P+++	Very nu- merous	None to
4 D.....	P+++	+	+	P++	..	+	Numerous	Strongly
5 D.....	P+++	Numerous	None
6 D.....	+	Numerous	None

of these 5, three cases gave evidence of very large numbers of this species. It is rather striking that there is not a single example of such a condition in the control cases. This would seemingly afford some basis for a reversal of the opinion that strongly putrefactive anaerobes are not concerned in this condition, especially in view of the fact that if one were to include the cases dominated by considerable numbers of *B. bifermentans* and *B. putrificus*, the instances of floras having proteolytic tendencies would be considerably increased. If these bacteria have no bearing on the causation of the morbid conditions presented in this disease, one may ask how it is possible to account for the numbers of proteophiles here present, in comparison with the controls. Before attempting to answer this question, it may be well to review first some of the more intimate biologic characteristics of the strongly and weakly

TABLE 4
PROTEOLYTIC ACTION OF COMBINED ANAEROBIC SPORE-BEARING FLORA ON COOKED MEAT MEDIUM

Cases	Non-proteolytic		Feebly Proteolytic		Strongly Proteolytic		Mixed		Total Number
	No.	%	No.	%	No.	%	No.	%	
Intestinal toxemia.....	8	44.4	4	22.2	5	27.7	1	5.5	18
Chronic diarrhea.....	2	33.3	2	33.3	1	16.5	1	16.5	6
Chronic eczema, etc.	3	37.7	3	37.7	2	25.0	0	0	8
Chronic arthritis.....	3	50.0	1	16.5	1	16.5	1	16.5	6
Perniciious anemia.....	24	100.0	0	0	0	0	0	0	24
Controls.....	5	50.0	3	30.0	0	0	2	20.0	10

proteolytic groups of anaerobes here isolated and see in what possible way, if at all, these types would be able to exert any harmful influence.

In the first place, although strongly proteolytic, *B. sporogenes* must be classed as nonpathogenic, from the experimental evidence now at hand. I have on several occasions, injected large quantities (2 or 2.5 c.c.) of an actively growing culture of the organism intraperitoneally into laboratory animals, without harmful or toxic effects. Other observers, Hall and Kendall, Day and Walker, have arrived at the same conclusion. Weinberg and Seguin claimed to have isolated a substance in filtrates obtained from young cultures of this species which produced rapid death when injected intravenously into rabbits. I am somewhat dubious about this observation in view of the fact that in my hands even the whole culture of *B. sporogenes*, when injected, has been constantly without harmful effect. It is quite possible that the French investigators were working with an impure strain, for as far as is

known, their results as stated above have not been duplicated. Hall tested several cultures of *B. sporogenes* on rabbits and guinea-pigs by both intravenous and subcutaneous inoculation, without harmful results. I, therefore, feel convinced that *B. sporogenes* is not a pathogenic species. It seems quite logical to assume that if this organism did elaborate any toxic principle during the process of its protein metabolism, such substances would become apparent on animal inoculation, especially when, as in these instances, massive doses were given. The cultures used for such experiments gave evidence that proteolysis had far advanced. On the other hand, Barget and Dale have noticed that when very old putrefactive cultures of *B. sporogenes* are injected into laboratory animals, anaphylactic-like symptoms may be produced. This action is ascribed by Hall to the production of ptomaine-like bodies and not to the elaboration of a specific toxin. The work of Barget and Dale does not, in any way, tend to substantiate the claims of Weinberg and Seguin who experimented with filtrates of very young cultures, 18 to 24 hours old.

In cases of intestinal toxemia, in which there is also severe constipation, it is well known that partially digested food residues may remain in certain localities of the large intestine for a considerable time. If the flora of such a person suffering from intestinal stasis contained large numbers of *B. sporogenes*, and in addition, if this hypothetical host subsisted on a diet composed largely of animal protein, there is presumably a vague possibility of some absorption of those ptomaine-like bodies taking place. *B. sporogenes* elaborates no indol, so that here, at least, this possibly toxic derivative of tryptophan is not increased in amount because of the activities of excessive numbers of this organism within the intestine, except perhaps indirectly through splitting up native protein and thus producing suitable pabulum for the indol formers.

Of course it can be argued that the failure to produce death or very noticeable organic disturbances when large amounts of fluid cultures of this highly proteolytic type are injected into laboratory animals does not entirely preclude an insidious and slowly progressive action which would not be manifest in inoculation experiments, but which might give rise to the symptoms displayed by persons said to be suffering from intestinal toxemia. Further, some may claim that the fact that substances elaborated by these putrefactive anaerobes are not toxic for lower forms of animal life does not entirely exclude the possibility that the higher cerebral centers of man are more easily affected by the absorption of small amounts of these putrefactive by-products. On the

basis of body weight, however, it must be assumed that a 1 or 2 c.c. dose of an actively putrefactive broth culture of *B. sporogenes* given intraperitoneally or intravenously into laboratory animals is a much larger proportional dose than could possibly be absorbed by a human being over a considerable period, and, if toxic principles were present, this relatively massive volume would produce an acute action in the animal if it were capable in much smaller dosage of inducing a chronic toxic effect in man.

If we include in this discussion the cases in the intestinal toxemia group showing a predominance of *B. bif fermentans* and *B. putrificus*, weakly proteolytic types, the number of cases having the anaerobic flora proteolytically disposed will be increased, but so also will those of the controls. Analysis of the biologic characteristics of these two species, the only members of the feebly proteolytic group found predominating, gives even less credence to the theories crediting them as being factors of importance in this disease. *B. bif fermentans*, as has been shown, is generally weakly proteolytic. It digests meat, egg albumin and inspissated serum at a much less rapid rate and not as completely as does *B. sporogenes*. No investigators, as far as we know, attribute even the most remote pathogenic properties to this species, although it forms indol and produces hydrogen sulphide. *B. putrificus* is even less marked in its ability to cleave protein. It does not, as a rule, form acid from any of the carbohydrates, so no irritating action could be attributed to it on that account. Observers are unanimous in attesting to its non-pathogenic and nontoxic qualities when injected into laboratory animals. Reddish and Rettger have recently published an account entirely substantiating this view. They found that a 1:10 suspension of a 2 weeks old egg-meat culture gave rise to no symptoms of intoxication whatever in guinea-pigs or white mice when injected intraperitoneally, although proteolysis had far advanced on this medium.

If one stops to consider the lack of pathogenic and toxic properties on the part of the proteolytic anaerobic spore-bearing bacteria isolated from a considerable number of cases in this series, we believe it would be distinctly indefensible, on the basis of the information afforded, to ascribe to them any direct connection with the morbid conditions supposedly arising from intestinal toxemia.

There must, however, be a reason for the dissimilarity of the results obtained in the control cases and in those of this group, as regards the occurrence of large numbers of these putrefactive anaerobes. It seems

not illogical through evidence furnished by case histories and bacteriologic data that inadequate digestion in some of these cases created by nervousness or a host of other conditions may cause a good deal of partially digested animal protein to be swept down into the large intestine. This condition, especially when coupled with chronic stasis, would afford excellent environment and source of energy for the propagation of the spore-bearing anaerobes possessed of strong protein-splitting ability.

Even if such conditions did exist and as a result the proteolytic species grew up in large numbers, on the basis of experimental data collected by others as well as myself, I see only one basis for concluding correlation, and that is through the possible assumption of certain obscure and unknown chemical interaction taking place between products elaborated by these protein-splitting types and other anaerobic spore-bearers and aerobic species present in the intestine. In this way there is a possibility of substances toxically inert in the test tube becoming potent and able to contribute toward exciting these pathologic conditions.

Caufield's ¹¹⁸ work seems to indicate that there may be some kind of bacteriologic interaction taking place between substances elaborated by one type of anaerobic spore-bearer and that of another. For example, he found that filtrates of *B. sporogenes* attenuated the toxic substances elaborated by *B. oedematiens* and *Vibrio septique*. If one desired to implicate any of the anaerobes as possible exciters of these toxemias, it would surely be logical to scrutinize closely the two latter named species on account of the potent poisons which they are able to elaborate. Caufield's findings, in fact, lend even less support to theories crediting strongly proteolytic spore-bearing anaerobes as possible factors in these toxemias, but rather could be interpreted as attributing a protective rôle to *B. sporogenes*, the most outstanding member of the strongly proteolytic anaerobic spore-bearing group. In this connection it is interesting to note that among the cases examined by us, of the 4 yielding either *B. oedematiens* or *Vibrio septique*, *B. sporogenes* was the dominant species in only 1.

ANAEROBIC SPORE-BEARING FLORA OF THE INTESTINAL TOXEMIA GROUP DOMINATED BY NONPROTEOLYTIC TYPES

By referring to table 2, it will be seen that *B. welchii* was present in the large majority of the cases of this group. Fifteen out of a total of 18 cases (88%) gave cultural evidence of the presence of this species

¹¹⁸ Jour. Infect. Dis., 1920, 27, p. 151.

which were distributed among the various fermentation groups, as shown in table 3. Those cases in the toxemia group having the anaerobic flora entirely dominated by nonproteolytic species numbered 8, or 40% of the total. *B. welchii* assumed this position on 6 occasions, while *B. tertius* was thus implicated twice. Thus domination by the nonproteolytic group occurred with greater frequency than domination by the strongly or weakly proteolytic anaerobes if taken separately.

Table 5 shows that as regards the control cases, 9 of them, or 90%, gave evidence of the presence of *B. welchii*, and in one person only was it present in large numbers. Seventy % of these controls showed *B. welchii* as the dominating species among the anaerobes. *B. tertius* and

TABLE 5
INCIDENCE OF ANAEROBIC SPORE-BEARING BACTERIA IN CONTROL CASES

Control Cases	<i>B. sporogenes</i>	<i>B. bitumentans</i>	<i>B. putrificus</i>	<i>B. tetanomorplus</i>	<i>B. aerofaciens</i>	<i>B. welchii</i> 1	<i>B. welchii</i> 2	<i>B. welchii</i> 3	<i>B. welchii</i> 4	<i>B. tertius</i>	<i>B. fallax</i>	Numerical Index	Proteolytic Action of Combined Anaerobic Flora
A	+	..	+	P++	Few	None to feebly
B	+	P++	Few	None
C	..	P++	+	+	..	Few	Feebly
D	+	P++	Few	None
E	..	P++	+	+	+	Numerous	Feebly
F	+	+	..	P++++	+	..	Very numerous	None to strongly
G	+	P++	Few	None
H	+	P++	Few	None
I	P++	..	+	Few	None
J	+	..	P++	+	Fek	Feebly

B. fallax were found on 5 occasions, but never in numerical superiority to the other types.

As regards the intestinal toxemia cases, these findings seem to indicate that the mere presence of *B. welchii*, even though it is the dominating species among the anaerobes, cannot have very much bearing on the subject. Many observers, in fact, have attested to its almost constant presence in the normal adult intestine (McNeal, Latzer and Kerr).

Going further, we see that from the quantitative standpoint, only 1 control case out of the 7 showing domination by *B. welchii* gave evidence of the presence of considerable numbers of this species, while in the intestinal toxemia group, of the 6 cases showing the anaerobic flora dominated by *B. welchii*, 3 revealed this organism in large numbers. The comparative percentages on this basis would be 14 to 50,

as regards the presence of large numbers of *B. welchii* in the control and intestinal toxemia cases. On this basis, intestines showing infection with a large number of these fermentative bacteria may well be scrutinized as possibly giving rise to untoward conditions. In the first place, one must bear in mind that just as there are different varieties of *B. welchii*, so also may there be differences in individual susceptibility, as far as intestinal permeability is concerned, to the toxic products which under certain circumstances may be elaborated by *B. welchii*. When the numbers of this species present in the large intestine become unduly large, not necessarily in proportion to the other anaerobes, but as an individual entity, there may be ground for suspecting pathologic activity on its part. As will be seen from the following considerations, if one were to implicate any of the spore-bearing anaerobes, it would be more logical to base such a hypothesis on the group of which *B. welchii* is an outstanding member than on the occurrence, even in large numbers, of the strongly or the feebly proteolytic species.

Practically all investigators working with *B. welchii* have attested to the toxicity of cultures or filtrates of young cultures when introduced into the blood stream. The pathologic picture produced when laboratory animals are thus treated is too well known to bear repetition of its description. The respiratory embarrassment leading to profound intoxication, and at times death, is familiar to most medical scientists. The typical gaseous lesions produced when suitable doses of *B. welchii* are injected intramuscularly is also a matter of common knowledge, as well as the ability of this species to produce potent hemolysins. Besides these toxic elements, *B. welchii* is able to elaborate on suitable culture medium, to a certain extent similar to partially digested food, relatively large volumes of gas and also butyric acid, the latter known to be an irritating agent, as Herter first surmised. We know also that certain strains of *B. welchii*, although not restricted to any one of the 4 groups, vary in their ability to produce one or another of these disturbing elements. Bearing these facts in mind, it may be surmised that when certain strains of *B. welchii* assume undue numerical proportions in the intestine of a susceptible person, it is not too illogical to conceive that one or more of these pathologic agents may be elaborated and produce morbid effects on the host.

The exact mechanism of such a process, if indeed it does constitute a factor in intestinal toxemia, must still remain a matter of conjecture until suitable technic of study has been worked out. It is not altogether impossible, however, that the production of large amounts of gas

increasing the pressure, combined with the irritating effect of butyric acid, might contribute toward making a susceptible intestine more highly permeable to the toxic principles elaborated by this and kindred species, such as *B. oedematiens*, *Vibrion septique*, and toxic strains of *B. coli* and *B. proteus*. The latter forms of anaerobes I have not found with sufficient frequency or numerical superiority to warrant a place in this discussion.

The older workers, Metchnikoff, Rettger, Tissier and others, classified *B. welchii* as a proteophile and attributed its toxic action, at least as far as the intestine is concerned, to by-products of its protein metabolism; its injurious influence was accordingly supposed to be analogous to that of *B. putrificus*, *B. sporogenes* and "*B. oedematis maligni*." We now know that the protein-splitting ability of *B. welchii* is practically nil and in my mind there is serious doubt as to whether or not it exists at all. Therefore, this species cannot be implicated on the grounds advanced to that effect, although it does elaborate its toxic element largely from nitrogenous sources.

B. tertius was found to be present in large numbers on 2 occasions in the intestinal toxemia cases and present on 3 occasions in the control cases, but not in large numbers. This species is not toxic or pathogenic in any sense of the word, and I cannot here attribute significance to it unless of an obscure katalytic nature.

Chronic Diarrhea, Series B.—This series of cases, while limited in number, serves possibly to illustrate one point; that is, that as far as the anaerobic spore-bearers are concerned, *B. welchii* need not be constantly associated with chronic diarrhea. As will be seen in the historical section, *B. welchii* aroused the suspicion of a number of men as possibly a causative agent of acute diarrhea.

From observing table 5, it will be noted that the anaerobic flora in this group of cases was about equally dominated by nonproteolytic and feebly proteolytic types, while one case showed the presence of a large number of *B. sporogenes*. *B. welchii* was dominant in one instance, and here only did it occur in numbers large enough to merit more than passing consideration.

In comparing the bacteriologic findings of this series of cases with those of the controls, I find, in the main, little difference. The most outstanding divergence seems to be in the fact that in one of the cases of chronic diarrhea the flora was dominated by *B. sporogenes* which, in this instance, occurred in considerable numbers. No such strongly

proteolytic anaerobic flora was to be found among the control cases. For characteristic cultural reasons already mentioned, it would indeed be difficult to attempt to correlate *B. sporogenes* with the ailment in question.

Kendall and Day claim that when proper conditions exist, *B. welchii* through the irritating action exerted by the elaboration of butyric acid may produce diarrhea even to the extent of blood, mucus and pus. In order to make such a condition possible, they hold two factors necessary: (1) An excess of carbohydrate must be present in the intestine of a type utilizable by *B. welchii*. (2) There must be a diminution of lactic acid forming bacilli, which they say are capable normally, through the production of this acid, of holding *B. welchii* in check. This indeed may be so, but from the evidence afforded in the cases here examined, it would be difficult to implicate *B. welchii*. As the fecal specimens were delivered to this laboratory just after an attack of diarrhea, it seems logical to suppose that if *B. welchii* were the causative agent, they would have been present in much larger numbers than the examination proved them to be.

As has been stated elsewhere, my view concerning the sensitivity of *B. welchii* to lactic acid is not in accord with those of Kendall and Day. It has been shown by Torrey¹¹⁹ that although *B. welchii* finds congenial surroundings in the intestine of dogs, the feeding of relatively large amounts of lactose or dextrin does not increase the numbers of this species. Lactose, dextrin, and, to a limited extent, saccharose, are the only carbohydrates so far reported capable of reaching the lower intestine in amounts sufficiently large to have any bearing on the problem of alteration of intestinal flora, and these are all utilizable by *B. welchii*. Of course, results obtained with dogs may not be exactly comparable to human beings, but this objection may be made to any series of animal experiments.

It is interesting to note that of the 6 cases examined in this series, 2 contained anaerobic spore-bearers known to be definitely pathogenic; namely, *B. fallax* and *B. oedematiens*. Although here these species have occurred in small numbers, it is not altogether inconceivable that, given proper environmental latitude, they may be more concerned in these untoward intestinal conditions than is now suspected.

It is thus seen that from the experimental data here gathered I am unable to correlate definitely chronic diarrhea in adults with the presence and growth of the anaerobic spore-bearing bacteria.

¹¹⁹ Jour. Med. Res., 1919, 39, 429.

Chronic Eczema and Urticaria, Series C.—Clinicians have long associated certain skin eruptions of otherwise unknown etiology with changes within the intestinal tract supposedly brought about by certain types of bacteria. Mathews states that the putrefactive processes in the intestine have a remarkable relation to the skin. Besides being in a measure responsible for the formation of pimples, pustules, acne and boils, he claims that a spotted skin is generally a sign of putrefaction going on within the digestive tube. According to Mathews, these conditions are brought about through the ability of certain types of bacteria to disintegrate amino acids. By the decomposition of cysteine and cystine, he claims, hydrogen sulphide is formed, and when absorbed in small quantities is presumably one of the facts contributing to anemia and the skin conditions found in the chronically constipated. He also implicates methyl and ethyl sulphide as well as indole and skatole.

In view of this and kindred assertions, a study of the 8 cases comprising this series was undertaken to determine whether or not putrefactive anaerobes would be found in the feces in sufficiently large numbers or with sufficient regularity to justify an attempt to connect them with these affections of the skin. It may be said that these patients were suffering with chronic skin eruptions of obscure origin, and the stool specimens were voluntarily sent to this laboratory by various clinicians with hopes that an analysis of the fecal flora would throw some light on the problem. If table 3 is consulted, it will become evident that only on 3 occasions did anaerobic bacteria occur in sufficient numbers to warrant calling them numerous, and of these 3 the species dominating 2 of the cases were nonproteolytic anaerobes, i. e., *B. welchii* and *B. tertius*. Neither of these species is able to elaborate indole, skatole, methyl sulphide or ethyl sulphide, although H_2S may be elaborated by *B. welchii*. The one case showing a rather large number of strongly proteolytic anaerobes proved to have an anaerobic flora composed chiefly of *B. centrosporogenes*. A 4th case, it will be seen, was dominated by *B. sporogenes*, but the numbers present were comparatively smaller.

From the point of view of the potentialities of the combined anaerobic flora it will be seen that 37% were strongly proteolytic, 37% were weakly proteolytic and about 25% were nonproteolytic. The total flora showing any proteolytic characteristics is about 75%. For the most part, the feebly proteolytic anaerobes were present in such small numbers that I hesitate to attach any importance to their presence in view

of the results obtained with the controls. *B. welchii* occurred in 75% of these cases but only once in large numbers, thus not differing to any great extent from the controls.

From the series of 8 cases studied, I see no evidence for attempting to correlate spore-bearing putrefactive anaerobes with these untoward conditions of the skin.

Chronic Arthritis, Series D.—Earlier investigators have attacked this problem along essentially different lines than the ones presented in this study. Most of the older work was done on acute articular rheumatism and as was pointed out in a review of the bibliography, attempts were made to correlate that disease with specific infection of the blood and joints with *B. welchii*. Later researches, especially those of Simonds, have tended to discredit these earlier studies in spite of the positive conclusions reached by Achalme as recently as 1913. Other opinions are set down at length in the discussion of the literature.

We have attacked this problem from an essentially different angle, not examining, as the observers referred to did, joint exudates or the blood but rather the fecal flora of the cases in this group in the hope of obtaining some correlation with the presence of excessive numbers of peculiar types of anaerobic spore-bearing bacteria.

The number of cases examined was limited to 6, but it must be said that in the series there was no case of acute articular rheumatism nor of the other types of arthritis known to be associated with acute infections, such as gonorrheal or streptococcic arthritis; that is to say, all of the cases belonged in the obscure group of chronic joint disturbances of unknown etiology.

Aside from the fact that anaerobic spore-bearers seemed to occur in somewhat larger numbers in cases of this series than in those discussed previously, there is little indication of the possibility of correlation. Table 6 shows that the anaerobic flora of 3 of the cases was dominated by nonproteolytic species, but in spite of this it will be seen from table 3 that *B. sporogenes* was found in large numbers on 3 occasions also. In the one case in which the flora was about equally divided between these two species, *B. welchii* first asserted itself and then *B. sporogenes*.

Thus, as far as the anaerobic spore-bearing organisms are concerned, it may be seen even from this small series that there is no constancy of type or species.

Pernicious Anemia, Series E.—Without doubt the most striking phenomenon encountered during the course of this entire study was the almost complete dominance of the anaerobic flora in the pernicious cases of *B. welchii*. Another interesting observation is shown in table 6 which proves that this species occurred almost invariably in very large

TABLE 6
RELATIVE NUMBERS OF ANAEROBIC SPORE-BEARING BACTERIA ISOLATED FROM CASES

Cases	Very Numerous		Numerous		Few		Very Few		None		Total Number
	No.	%	No.	%	No.	%	No.	%	No.	%	
Intestinal toxemia.	3	16.6	7	39.3	7	39.3	0	0	1	5.5	18
Chronic diarrhea...	1	16.5	1	16.5	2	33.3	2	33.2	0	0	6
Chronic eczema, etc.	0	0	3	38.5	2	25.0	3	38.5	0	0	8
Chronic arthritis...	1	16.5	4	66.6	1	16.5	0	0	0	0	6
Pernicious anemia...	16	66.6	6	25.0	1	4.0	1	4.0	24
Controls.....	1	10.0	1	10.0	8	80.0	0	0	0	0	10

numbers. Sixty-six % of these cases exhibited a high *B. welchii* count, while 25% gave evidence of having this species present in considerable numbers. In only 2 patients was *B. welchii* encountered in small numbers, and in both of these instances the patients in question had early cases of pernicious anemia. These persons have since become considerably worse, and a corresponding large increase in the *B. welchii* count has taken place. It may be said of these 2 cases that even in the early period of the ailment, when the anaerobic count was much lower, *B. welchii* has occurred in such large comparative majority that the presence of other types of anaerobes has been almost entirely masked. Coate's theories are based on the assumption of the activities of proteolytic bacteria. This may or may not be true as regards certain aerobes, but on the basis of this investigation and the work of Herter, Rettger and Simonds, is it not more logical closely to scrutinize *B. welchii*, a nonproteolytic anaerobic species?

Control Cases.—From a study of the anaerobic spore-bearing bacteria contained in the fecal material of normal persons it is seen that rather a wide variety of species of this group may exist in the intestinal tract of human beings without giving rise to any disturbance of health. It also seems that under normal physiologic conditions of the human digestive system conditions are established which are somewhat more congenial to the propagation of anaerobes of the nonproteolytic group than of the proteolytic. Bacteria possessing strong protein-cleaving

ability have not been found to have occurred in large numbers. Representatives of the feebly proteolytic group have at times been found to occupy a position numerically superior to other species, and it is also seen that appreciable numbers of this type of micro-organism may be present in the normal intestine without occupying the dominating position in the anaerobic spore-bearing flora. Thus, the larger majority of the anaerobic species of the control cases, if we are at liberty to judge from test-tube reaction, seem to have a nonproteolytic tendency, typified by the presence of such types as *B. welchii*.

SUMMARY

This investigation represents an effort to review the possible relationship of anaerobic spore-bearing bacteria as they occur in the intestine, to certain clinical manifestations.

Intestinal toxemia (18 cases): Strongly proteolytic spore-bearing anaerobes (*B. sporogenes*) were found in larger numbers but with the same frequency in these cases as in the controls. It does not seem likely that they could be directly concerned in these intoxications, principally on account of their low pathogenicity.

Feebly proteolytic anaerobic spore-bearing bacteria have been found with almost identical incidence in control and intestinal toxemia cases. No significance, accordingly, has been attached to their presence.

B. welchii was found in larger numbers in this series of cases as compared with the controls. On the basis of their biologic activity, they may well bear closer scrutiny as possible factors in this condition.

Chronic Diarrhea (6 cases): No evidence has been found to justify an attempt to correlate anaerobic spore-bearing bacteria with this disease in adults. *B. welchii*, as has been generally assumed, was not found to be a constantly dominating species.

Chronic Eczema and Urticaria (8 cases): These conditions have at times been associated by other observers with putrefactive intestinal conditions. Half of these cases were found to have had nonproteolytic anaerobic spore-bearing intestinal flora.

Chronic Arthritis (6 cases): The spore-bearing anaerobes occurred in this series of cases in somewhat larger numbers than in the controls. There has been little constancy in the species isolated, however.

Pernicious Anemia (24 cases): In these cases, *B. welchii* occurred in the intestinal content in large numbers and almost always to the exclusion of other species of anaerobic spore-bearers. The strains of *B.*

welchii isolated were distributed throughout the 4 fermentative groups with no indication that one particular strain was peculiar to the disease. These findings justify a more detailed study of the possible primary or secondary etiologic association of *B. welchii* to pernicious anemia; this is now being carried on.

Control Cases (10): The majority of control cases exhibited a nonproteolytic anaerobic spore-bearing flora, *B. welchii* being the most frequently encountered species although usually occurring in smaller numbers than in some of the pathologic series. Strongly proteolytic species were found not to dominate the intestinal flora of the controls. Weakly proteolytic types were found to be present and at times in considerable numbers.

The following species were not isolated from any of the 65 cases studied: *B. histolyticus*, *B. botulinus*, *B. butyricus*, *B. sphenoides*, *B. chauvœi*. The following pathogenic species have been isolated: *B. welchii*, *Vibrio septique*, *B. oedematiens* and *B. fallax*, but with the exception of *B. welchii* on rare occasion. This suggests, however, that in case of war on native soil complicated types of gas gangrene similar to those occurring during the Great War, would be likely to be encountered.

A technic is described suitable for isolating and identifying anaerobic spore-bearing bacteria from intestinal sources.

FACTORS INFLUENCING THE DESTRUCTION OF URIC ACID BY AEROBACTER AEROGENES

E. E. ECKER AND J. LUCIEN MORRIS

From the Departments of Pathology and Biochemistry, School of Medicine of Western Reserve University, Cleveland, Ohio

We have shown that certain micro-organisms in simple mediums utilize uric acid as a source of nitrogen.¹ *Bacillus acidii urici* of Ulpiani² was isolated from chicken excreta and studied. The absence of a definite uricase indicated that the destruction is due to some metabolic activity. Of several other bacteria investigated, *Aerobacter aerogenes* exhibited comparable propensities. Because of the common occurrence of the latter organism in the intestinal canal, its capacity to utilize uric acid as a source of nitrogen, and the fact shown by us that after ingestion of uric acid considerable quantities are destroyed in the alimentary tract, we thought it of interest to study conditions of stimulation and inhibition. In the course of this study, we recognized certain chemical factors in the destruction of uric acid, some of which no doubt play a part in the destruction of this substance in the alimentary canal.

It has been customary by means of nonpurine diets to study the uric acid problem from the standpoint of endogenous metabolism. Valuable observations have been made, and recently most of the results have been coordinated by Folin, Berglund and Derick³ into the first real attempt to establish a working hypothesis of the mechanism of destruction and elimination of this product. Beyond the fact that ingested purine substances are undoubtedly precursors of uric acid which must be destroyed or eliminated by the body, there is little known of the extent of their digestion and the stage at which absorption takes place before they contribute the so-called exogenous uric acid to that which arises from the body itself. In addition to these variables, exogenous uric acid is subject to the degree of destruction which the intestinal flora is able to produce. Wells⁴ suggested that some of the food purines may be destroyed by bacteria in the intestines. Our early work indicated destruction of uric

Received for publication, June 9, 1924.

¹ Jour. Infect. Dis., 1924, 34, p. 592.

² Gaz.-Chem., 1903, 33 (2), p. 93.

³ Jour. Biol. Chem., 1924, 60, p. 361.

⁴ Jour. Lab. & Clin. Med., 1915, 1, p. 164.

acid itself in fecal infusions, and this report deals with an organism, isolated from feces, displaying a remarkable avidity for this substance.

Technic.—Five strains of *Aerobacter aerogenes* were isolated from water and one from milk. These organisms inoculated in Koser's medium destroyed, respectively, 19, 12, 44, 17, 12 and 11% of the uric acid present in 48 hours at 37 C. Except one fecal strain (F_4), which was motile, all were gram-negative, encapsulated and nonmotile. On agar their colonies were thick, spreading and white. Litmus milk was coagulated with acid formation. Dextrose, lactose and sucrose were readily fermented. Gas formation was in the correct ratio. All these organisms grew well in Koser's uric acid and citrate mediums. The fecal strains gave a negative methyl red and an atypical Voges-Proskauer reaction. F_2 caused death of a guinea-pig, and smears made from the animal revealed a beautiful capsule. Three strains were employed in the experiments n1: W_3 , W_5 and F_2 . The first two being water strains and the last a fecal strain, W_3 destroyed considerably more uric acid than W_5 in the same period of incubation. For inoculation, a well shaken 18 to 24-hour Koser medium culture was used; 0.1 c.c. was added to 5 c.c. of the medium to be inoculated. Koser's ⁵ medium formed the basis of all our experiments. The quantity of uric acid present was 200 mg. per liter instead of 500 mg. used by Koser. The smaller amount facilitated observations on the rate of utilization. The composition is as follows:

Fresh distilled water.....	1,000 c.c.
NaCl	5.0 gm.
MgSO ₄	0.2 gm.
CaCl ₂	0.1 gm.
K ₂ H PO ₄	1.0 gm.
Glycerol	30.0 gm.
Uric acid	0.2 gm.

The medium was distributed in test tubes of uniform diameter of about 15 x 1.5 cm. and in exactly 5 c.c. amounts. Sterilization was done in the autoclave at 15-20 lbs. for 5-10 minutes. The P_H fluctuated between 6.6 and 6.7, determined electrometrically. When no immediate analysis was possible, the cultures were killed with chloroform and stored in the icebox at 3-4 C. All determinations were quantitative by the Morris and Macleod ⁶ technic, but preliminary precipitation as zinc

⁵ Jour. Infect. Dis., 1918, 23, p. 377.

⁶ Jour. Biol. Chem., 1922, 50, p. 55.

urate was omitted. In order to control possible destruction by sterilization, addition of various chemicals and incubation at 37 C., parallel analysis of a blank series was always made and the P_H also determined.

To demonstrate clearly the effect of substances added to the medium, we employed strain W_5 in our first experiment, because this organism destroyed only 12% of the uric acid present in 48 hours at 37 C. It will be observed in some of the tables that the amounts normally destroyed in Koser's medium varied. Increased destruction in controls indicates adaptation of the organism to the medium.

As shown in table 1, glycine at first has a mild, stimulating influence, but the destruction progresses slowly at the end of 48 hours; the

TABLE 1
EFFECT OF VARIOUS SUBSTANCES ON THE DESTRUCTION OF URIC ACID BY AEROBACTER
AEROGENES (STRAIN W_5) IN KOSER'S MEDIUM

All Substances Added in 0.5% Amounts	Incubation at 37 C. and Amount Destroyed				
	24 Hours	48 Hours	72 Hours	96 Hours	Control, 96 Hours
	Percentage	Percentage	Percentage	Percentage	Percentage
Koser (sterile control).....	0	0	0	0	0
Koser (culture control).....	0	17	39	46	2
Glycine.....	23	31	37	36	18
Stearic acid.....	2	13	62	61	0
Sodium acetate.....	6	10	18	19	4
Sodium benzoate.....	3	4	2	2	1
Sodium citrate.....	10	24	69	87	6
Sodium glycocholate.....	17	22	36	26	20
Sodium oxalate.....	2	66	86	100	13
Sodium salicylate.....	4	7	8	30	11
Sodium tartrate.....	0	17	61	..	2

amount destroyed in 96 hours being only 5% more than that in the 48-hour culture. Considerable destruction of uric acid occurred following the introduction of this amino acid. Sodium citrate, sodium oxalate, and sodium tartrate definitely enhanced destruction. When the control medium showed only 46% destruction in 96 hours, the presence of sodium citrate produced 87% and sodium oxalate 100% destruction in the same time. At the end of 72 hours, the medium containing sodium tartrate also showed 61% destruction against 39%. Sodium benzoate, sodium acetate and sodium salicylate definitely inhibited, while stearic acid, not very soluble, exhibited, if any, a mildly stimulating effect.

In a subsequent experiment, the minimum stimulating amount of three of the salts was studied and also the effect of ammonium salts (table 2). A maximal destruction occurred when sodium citrate was

used in 0.15% amounts. The optimal effect was obtained with sodium oxalate in 0.1% doses. Here a little variation occurred in that the 30-hour culture did not show as great a reduction as the 24-hour culture.

TABLE 2
EFFECT OF SMALL AMOUNTS OF SODIUM CITRATE, SODIUM OXYLATE, AND SODIUM TARTRATE ON THE DESTRUCTION OF URIC ACID BY AEROBACTER AEROGENES (STRAIN W₃) IN KOSER'S MEDIUM *

Amounts Used	Incubation at 37 C. and Amount Destroyed				
	12 Hours	18 Hours	24 Hours	30 Hours	Control,
	Percentage	Percentage	Percentage	Percentage	30 Hours Percentage
Sterile control.....	0	0	0	0	0
Culture control.....	8	13	13	21	2
0.05% sod. citrate.....	6	13	17	29	2
0.10% sod. citrate.....	9	10	38	34	3
0.15% sod. citrate.....	8	6	26	41	5
0.20% sod. citrate.....	7	13	32	35	4
0.05% sod. oxalate.....	8	12—	23	27	3
0.10% sod. oxalate.....	11	18	53	33	8
0.15% sod. oxalate.....	9	15	42	41	5
0.20% sod. oxalate.....	10	15	32	30	4
0.05% sod. tartrate.....	11	15	29	33	2
0.10% sod. tartrate.....	13	12	29	38	3
0.15% sod. tartrate.....	..	15	30	33	4
0.20% sod. tartrate.....	12	16	57?	44	6
Destruction of Uric Acid Inhibited by Ammonium Salts					
0.10% (NH ₄)Cl.....	9	8	9	16	5
0.20% (NH ₄)Cl.....	6	10	7	19	4
0.10% (NH ₄) ₂ SO ₄	6	10	9	13	6
0.20% (NH ₄) ₂ SO ₄	8	10	10	18	2

* Excellent growth in ammonium chloride and sulphate tubes.

TABLE 3
EFFECT OF VARIOUS SUBSTANCES ON THE DESTRUCTION OF URIC ACID BY AEROBACTER AEROGENES (STRAIN W₃) IN KOSER'S MEDIUM

All Substances Added in 0.5% Amounts	Incubation and Amounts Destroyed			
	14 Hours	24 Hours	48 Hours	Control,
	Percentage	Percentage	Percentage	48 Hours at 37 C. Percentage
Koser sterile control.....	0	0	0	0
Koser culture control.....	5	11	76	4
Acid glycollic.....	7	40	78	78
Acid hippuric.....	13	67	67	71
Acid lactic.....	4	62	78	67
Acid pyruvic.....	13	40	70	78
Feces.....	0	9	75	—3
Glucose.....	0	—3	19	—7

The maximal effect of sodium tartrate was found in the tube containing 0.2%. From the results secured with ammonium salts we may safely conclude that the organisms preferably attack the ammonium salts.

Growth was extensive in those tubes. Certain organic acids lowered the P_H to <5 , inhibiting the organism (table 3). As shown, the fecal extract neither stimulated nor inhibited destruction. In the presence of glucose, there was little destruction of uric acid, probably due

TABLE 4

EFFECT OF VARIOUS SUBSTANCES ON THE DESTRUCTION OF URIC ACID BY AEROBACTER AEROGENES (STRAIN W_3) IN KOSER'S MEDIUM

All Substances Added in 0.2% Amounts	Incubation at 37 C. and Amount Destroyed				
	12 Hours Percentage	24 Hours Percentage	36 Hours Percentage	48 Hours Percentage	Control, 48 Hours Percentage
Koser sterile culture.....	0	0	0	0	0
Koser culture control.....	10	56	Trace ± 100	100	0
Alanine.....	10	5	7	71	0
Cystine.....	17	10	10	4	13
Histidine HCl.....	12	32	50	62	0
Pyrrole.....	7	28	73	100	6
Scatole.....	5	9	3	7	4
Tryptophane.....	12	25	86	100	8
Tyrosine.....	6	50	86	89	2
Sodium bicarbonate.....	21	22	46	52	33
Sodium butyrate.....	19	45	78	100	13

TABLE 5

EFFECT OF VARIOUS SUBSTANCES ON THE DESTRUCTION OF URIC ACID BY AEROBACTER AEROGENES (STRAIN W_3) IN KOSER'S MEDIUM

All Substances Added in 0.5% Amounts	Incubation at 37 C. and Amount Destroyed				
	12 Hours Percentage	24 Hours Percentage	36 Hours Percentage	48 Hours Percentage	Control, 48 Hours Percentage
Koser sterile control.....	0	0	0	0	0
Koser culture control.....	4	25	85	87	0
Sodium glycollate.....	9	26	61	100	0
Sodium hippurate.....	10	18	25	26	3
Sodium lactate.....	5	57	85	100	0
Sodium pyruvate *.....	35	37	88	67	69
Substances Added in 0.2% Amounts					
Sodium glycollate.....	7	41	100	100	1
Sodium hippurate.....	2	12	18	19	0
Sodium lactate.....	5	33	86	100	2
Sodium pyruvate.....	70	33	84	83	72
Glycine.....	16	41	48	62	9

* Precipitates uric acid.

to sparing action. The apparent increase of uric acid, in the 24-hour tube is even greater in the 48-hour control, and, as noted in our earlier paper, was due to the formation in small amounts of oxidation products of glucose which react with the arsenotungstate complex. As seen in table 4, alanine has an inhibiting effect. It yields nitrogen. Cystine

proved to be inhibitory, while histidine-HCl paralleled alanine. Scatole inhibited, pyrrole, tryptophane, tyrosine and sodium butyrate were without effect. Sodium bicarbonate inhibited the growth of the organism although the uric acid was at the same time destroyed at a fair rate. The amount of destruction in the bicarbonate sterile control indicates that the bicarbonate itself is in large part responsible for this, a point to which we refer subsequently.

It was found that sodium lactate, 0.5% and 0.2%, definitely enhanced destruction of uric acid in the first 24 hours of growth of the organism (table 5). Sodium hippurate inhibited while sodium glycollate had no action.

TABLE 6
EFFECT OF SODIUM BICARBONATE ON THE DESTRUCTION OF URIC ACID BY AEROBACTER
AEROGENES (STRAIN F₂) IN KOSER'S MEDIUM *

Amounts of Sodium Bicarbonate	Incubation at 37 C. and Amount Destroyed	
	24 Hours Percentage	48 Hours Percentage
Koser control sterile.....	0	0
1% sod. bicarb. sterile control.....	0	0
1% sod. bicarb. culture.....	14	21
2.5% sod. bicarb. sterile control.....	14	16
2.5% sod. bicarb. culture.....	45	59
5.0% sod. bicarb. sterile control.....	22	27
5.0% sod. bicarb. culture.....	62	100
10% sod. bicarb. sterile control.....	28	35
10% sod. bicarb. culture.....	77	100

* It was possible to isolate F-2 from 2.5% sodium bicarbonate Koser's medium, but growth is inhibited at 0.5%. The organism readily dies in these concentrations of sodium bicarbonate. The P_H of this medium was ± 9 by the colorimetric method.

The marked destruction of uric acid incubated in sterile tubes with sodium bicarbonate is probably due to an increased sensitiveness to oxidation, previously described and made use of by one of us⁷ as a basis for a selective oxidation determination of uric acid. This peculiar behavior of bicarbonate and the additional fact that it is normally present in the intestines led us to a more detailed study of the effect of various concentrations of bicarbonate on the utilization of uric acid by *Aerobacter aerogenes*, and on the chemical destruction of this substance. The results of one such experiment are given in table 6. The presence of 2.5% or larger amounts of sodium bicarbonate led to considerable

⁷ Morris, J. L.: *Ibid.*, 1919, 37, p. 231.

destruction of the acid. The organism was inhibited when 0.5% of the salt was employed, but we were still able to reculture the organism at the end of 24 hours from the tube containing 2.5% of sodium bicarbonate. Although growth was inhibited, the presence of the organism evidently contributed to the destruction. At the end of 48 hours, we were unable to recover the organism. A count made of cultures containing 1, 2.5, 5 and 10% sodium bicarbonate showed that while about 3,330,000,000 per c c. developed in the Koser's medium following transplantation of the organism on this medium, about 25,000,000 developed in the presence of 1% sodium bicarbonate, 1,220 in the presence of 2.5% and only 2 when 5% was present. Plates made from the 10% sodium bicarbonate medium remained sterile.

TABLE 7

EFFECT OF HEIGHT OF COLUMN AND ACCESS TO AIR ON THE DESTRUCTION OF URIC ACID IN KOSER'S MEDIUM. INCUBATION 48 HOURS AT 37 C. AMOUNT DESTROYED GIVEN IN PERCENTAGES

Amounts of Sodium Bicarbonate, Percentage	Open Controls, Percentage	Rubber Stoppered Controls, Percentage	Open Tube Cultures, Percentage	Rubber Stoppered Cultures, Percentage
1	46	43	47	28
2.5	54	50	49	43
5	52	57	57	60
10	41	49	60	50

Of the possible explanations of this acceleration of uric acid destruction we considered it plausible that during the brief period in which the organism lived an enzyme might have been produced which after death of the organism served as a catalyst in the destruction of uric acid. Our attempts to test this hypothesis have furnished inconclusive results, owing to the action of the more definite and apparently more effectual factor of exposure to air.

Table 7 presents results with closed and open tubes, both with and without sodium bicarbonate. When the test tubes were filled with Koser's medium and closed with a rubber stopper, destruction progressed about the same as in the open tubes. Presence of the culture caused no added destruction. When the organisms were grown in high columns of Koser's medium not containing sodium bicarbonate, destruction took place at a slower rate, requiring at least 72 hours for destruction of 78% of the acid. At this time, the organism had been

transferred 11 times in Koser's medium, and its growth was rapid (table 8). Layering of the medium with paraffin oil also inhibited destruction.

TABLE 8
EFFECT OF HIGH COLUMNS OF KOSER'S MEDIUM WITH AND WITHOUT A LAYER OF PARAFFIN OIL ON THE DESTRUCTION OF URIC ACID BY AEROBACTER AEROGENES

Testtubes 15 × 1.5 cm. Columns of liquid 8 cm., oil 1.5 cm.			
	Incubation at 37 C. and Amounts Destroyed		
	24 Hours Percentage	48 Hours Percentage	72 Hours Percentage
Control.....	9	8	4
Culture.....	13	32	78
Control and oil.....	11	7	7
Culture and oil.....	11	7	7

TABLE 9
SURFACE AREA, A FACTOR IN THE DESTRUCTION OF URIC ACID IN THE PRESENCE OF SODIUM BICARBONATE (STRAIN F₂). 20 C.C. WERE PLACED IN WIDE BOTTLES OF 5 CM. DIAMETER

Amount of Sodium Bicarbonate Used in Koser's Medium	Incubation at 27 C. and Amount Destroyed		Remarks
	24 Hours Percentage	48 Hours Percentage	
Koser sterile control.....	0	0	
1% sod. bicarb. not autoclaved.....	6	..	
1% sod. bicarb. autoclaved.....	14	29	
2.5% sod. bicarb. not autoclaved.....	11	..	
2.5% sod. bicarb. autoclaved.....	24	78	
5% sod. bicarb. not autoclaved.....	17	..	
5% sod. bicarb. autoclaved.....	31	90	
10% sod. bicarb. not autoclaved.....	23	..	
10% sod. bicarb. autoclaved.....	32	100	
1% sod. bicarb.	18	28	Inoculated with Aerobacter aerogenes F-2
2.5% sod. bicarb.	43	67	Inoculated with Aerobacter aerogenes F-2
5% sod. bicarb.	62	83	Inoculated with Aerobacter aerogenes F-2
10% sod. bicarb.	65	100	Inoculated with Aerobacter aerogenes F-2
1% sod. bicarb.	23	37	In this series the cultures were chloroformed after 24 hours at 37 C. incubation
2.5% sod. bicarb.	44	65	
5% sod. bicarb.	62	89	
10% sod. bicarb.	66	100	

Table 9 includes the results of a series in which large surface area and the action of Aerobacter aerogenes were both used in the destruction of the acid. Considerable destruction took place in the bicarbonate bottles following the autoclaving and sufficient exposure of the

medium to air. In 48 hours and the presence of 10% sodium bicarbonate, 100% of the acid was destroyed. The same result was obtained when *Aerobacter aerogenes* was inoculated in a parallel series, indicating the destruction to be due to sodium bicarbonate and sufficient access to air. In a third series, the organism was chloroformed at the end of 24 hours' growth, and here again destruction continued due to the bicarbonate function. It was, therefore, definitely established that sufficient air and large concentrations of sodium bicarbonate lead rapidly

TABLE 10
EFFECT OF SODIUM OXALATE ON THE INHIBITORY INFLUENCE OF SODIUM BICARBONATE
DURING THE COURSE OF DESTRUCTION OF URIC ACID BY *AEROBACTER AEROGENES*
(STRAIN F₂) IN KOSER'S MEDIUM

Amount of Sodium Oxalate Used	Incubation at 37 C. and Amount Destroyed		
	12 Hours Percentage	24 Hours Percentage	Control, 24 Hours Percentage
Koser sterile control.....	0	0	0
Koser culture control.....	46	94	..
0.1% sod. bicarb.	18	67	26
0.2% sod. bicarb.	19	..	32
0.05% sod. oxalate.....	55	100	12
0.1% sod. oxalate.....	46	100	19
0.15% sod. oxalate.....	59	..	18
0.2% sod. oxalate.....	48	100	13
0.1% sod. bicarb. + 0.05% sod. oxalate.....	30	67	27
0.1% sod. bicarb. + 0.1% sod. oxalate.....	29	22	30
0.1% sod. bicarb. + 0.15% sod. oxalate.....	22	20	28
0.1% sod. bicarb. + 0.2% sod. oxalate.....	24	23	26
0.2% sod. bicarb. + 0.05% sod. oxalate.....	21	21	33
0.2% sod. bicarb. + 0.1% sod. oxalate.....	21	30	33
0.2% sod. bicarb. + 0.15% sod. oxalate.....	25	33	35
0.2% sod. bicarb. + 0.2% sod. oxalate.....	29	36	38

to complete destruction of uric acid. However, reference to the figures for 24 hours shows just as definitely that although the destruction in the culture tubes follows the same order of the bicarbonate controls, the degree of destruction is in every case increased (almost doubled) by the presence of organisms. The surface area factor is thus an obvious hindrance to any inquiry as to the nature of the organism's effect. All attempts to prove it enzymatic have so far failed.

It was then thought of interest to study whether or not the stimulating organic salts may counteract the inhibitory mechanism of sodium bicarbonate. In this experiment, smaller doses of sodium bicarbonate were used and optimal doses of the stimulating substances. The experiment in table 10 proves that sodium oxalate failed to produce the desired effect. Similar experiments were made with sodium citrate.

CONCLUSIONS

The utilization of uric acid by *Aerobacter aerogenes* is stimulated by sodium citrate, sodium oxalate, sodium tartrate and sodium lactate, each according to its optimal concentration.

Amino acids and ammonium salts inhibit uric acid destruction by *Aerobacter aerogenes* probably because their nitrogen is in a more available form. Glucose also inhibits on account of its nitrogen sparing action.

Sodium bicarbonate exerts a marked inhibitory effect. It inhibits the growth of *Aerobacter aerogenes*. In certain concentrations this salt destroys uric acid regardless of the presence of *Aerobacter aerogenes*. This action is apparently a chemical one and probably an oxidative phenomenon.

Sodium citrate and sodium oxalate failed to counteract the inhibitory properties of sodium bicarbonate.

These observations indicate methods by which uric acid in the intestinal tract is eliminated.

THE ABORTIN REACTION IN THE TESTICLE AS AN INDICATOR OF THE HYPERSENSITIVENESS OF INFECTION *

MAC HARPER SEYFARTH

*From the Department of Pathology of the University of Chicago and the Otho S. A. Sprague
Memorial Institute*

The purpose of this paper is to report further investigation of the hypersensitiveness of infection with *B. abortus* by means of the testicular abortin test. The reader is referred to recent articles by Long¹ and Long and Seyfarth² on the use of the testicle as an extremely sensitive indicator of the hypersensitive state in the tuberculous guinea-pig. In the course of the investigations reported in their papers, the abortus experiment was tried on a small scale. The guinea-pigs were injected intraperitoneally, and it was found that the lesions of abortus infection itself produced by this means of infection were extensive and especially prone to attack the testicle. The effect of abortin on the testicle of the guinea-pig infected with *B. abortus* was thus not as clear cut as that of tuberculin on the tuberculous pig.

In this article, the terms abortus and melitensis, and abortin and melitensin, have been used interchangeably. The basis of this terminology lies in the work of Evans³ in which she showed "that morphologically, serologically, and biochemically the *B. abortus bovinus* and the organism which causes undulant or Malta fever are practically indistinguishable." She showed that *B. abortus* and *B. melitensis* were identical on the various culture mediums; that the biochemical changes of the two organisms were the same and that the agglutination reactions to a *B. abortus* antiserum were exactly alike. In carrying out a set of absorption tests, it was found, furthermore, that for practical purposes using a *B. abortus* antiserum, *B. abortus* and *B. melitensis* cannot be distinguished from one another. When application was made to the United States Hygienic Laboratory for a culture of *B. abortus*, a culture of *B. melitensis* of Bruce was forwarded to this laboratory. Obviously, the organisms were considered as identical.

Received for publication, July 1, 1924.

* Aided by the National Tuberculosis Association grant to Dr. E. R. Long for studies on the chemistry of the tubercle bacillus and the nature of the tuberculin reaction.

¹ Am. Rev. of Tuberc., 1924, 9, p. 215.

² Ibid., p. 254.

³ Jour. Infect. Dis., 1918, 22, p. 580.

The term "hypersensitiveness of infection" is limited as defined by Coca⁴ recently. The best known example of this type of hypersensitiveness is that observed in tuberculosis, but the abortin, mallein and typhoidin reactions appear to be of identical type. This type of allergic state is distinguished from the anaphylactic condition in that it appears to be elicited only by actual infection with living organisms.

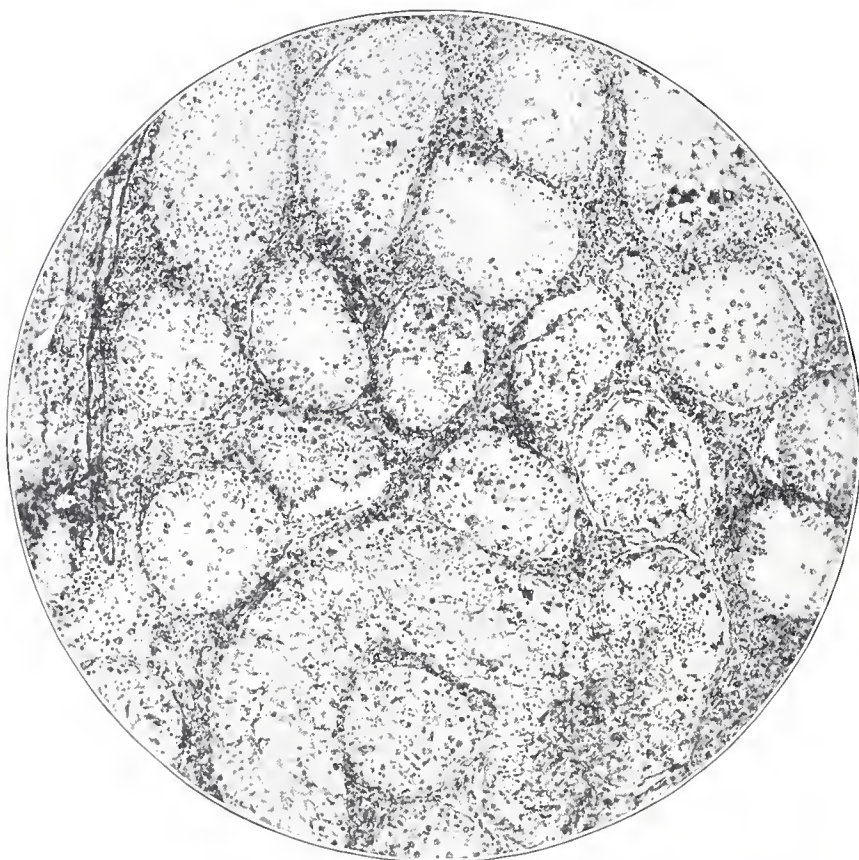


Fig. 1.—Abortion reaction in the testicle. Section of left testicle of guinea-pig E1 ($\times 60$). First injection, subcutaneous, with 1 c.c. of a saline suspension of *B. abortus* in the left axilla, Jan. 31, 1924. Second injection, intratesticular, with 0.1 c.c. of abortin, March 8, 1924. Guinea-pig killed 48 hours later. This testicle was much swollen and hyperemic grossly. This swelling can be observed microscopically by the marked interstitial edema, with pronounced wandering cell infiltration. Note the decided degeneration of the germinal cells. There is still some spermatogenesis. These are the typical early results of the abortion reaction.

Krause has repeatedly emphasized this point. It has long been known, however, that a transient hypersensitiveness can be induced with dead bacteria, and recent observations by Zinsser and Petroff⁵ indicate that

⁴ Jour. Immunol., 1923, 8, p. 163.

⁵ Jour. Immunol., 1924, 9, p. 85.

the sensitive state so induced lasts longer than was formerly supposed. Fleischner, Meyer, and Shaw ⁶ also have given a concise report on the hypersensitiveness of infection, with special reference to the abortus infection.

Baldwin ⁷ showed in his fundamental work that typical and lasting hypersensitiveness of the tissues of the body to tuberculin could be



Fig. 2.—Control to Fig. 1—abortin in normal animal. Section of left testicle of guinea-pig F1 ($\times 60$). Normal guinea-pig injected with 0.1 c.c. of abortin in the left testicle, March 8, 1924. Guinea-pig killed 48 hours later. Note the absence of any interstitial change as compared with Fig. 1; note also the active spermatogenesis. This testicle shows the absence of effect of abortin injected into a nonsensitized animal. The testicle is normal.

obtained only when the animal possessed an actual infection with living tubercle bacilli. After the infection has occurred, the tissues of the body respond with an acute inflammation following the introduction of the infecting agent or the products derived from it. This

⁶ Am. Jour. Dis. Child., 1919, 18, p. 577.

⁷ Jour. Med. Res., 1910, 22, p. 189.

sensitiveness, as Krause⁸ has pointed out, develops simultaneously with the development of the initial focus, increases progressively with the lesions, varies directly with the extent and intensity of the infection, and diminishes with healing. Baldwin attempts to explain this sensitization of the body cells by assuming that in the disintegration of the infective agent caused by body cells or fluids some product is set free



Fig. 3.—Late results of abortin reaction. Section of left testicle of guinea-pig C2 ($\times 60$). First injection, subcutaneous, with 2 c.c. of a saline suspension of *Bacillus abortus* in the left axilla, Jan. 31, 1924. Second injection, intratesticular, with 0.1 c.c. of abortin, Feb. 27, 1924. Guinea-pig killed 50 days later. Grossly, this testicle was much reduced in size. Note the extreme atrophy of the tubules with interstitial fibrosis and wandering cell infiltration. There remains, however, an occasional area showing primary spermatocytes.

in the blood which acts on the distant cell groups not connected with the specific lesions produced nor even in contact with the bacilli.

The strain of *B. abortus* or *B. melitensis* used in these experiments proved to be a highly pathogenic organism for guinea-pigs and produced

⁸ Trans. Twelfth Ann. Meeting Natl. Tuberc. Assn., 1916. p. 233.

a rapid and extensive type of lesion, a desirable condition for this kind of experiment. Three sets of male guinea-pigs weighing approximately 400 gm. were used. Four pigs were inoculated intraperitoneally with 1 c.c. of a 72-hour growth of *B. abortus* on veal infusion broth, 3 were inoculated in the left axilla with 2 c.c. of a suspension obtained from two 72-hour glycerol agar slants, while 4 more were inoculated in the

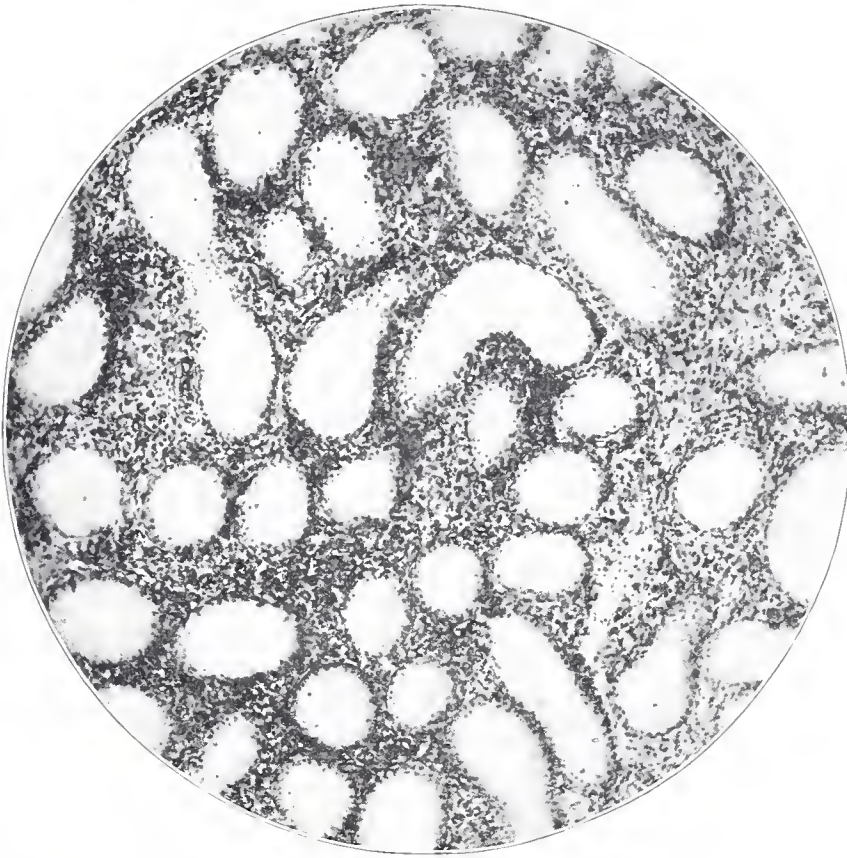


Fig. 4.—Late results of abortin reaction. Section of left testicle of guinea-pig E3 ($\times 60$). First injection subcutaneous, with 1 c.c. of a saline suspension of *Bacillus abortus* in the left axilla, Jan. 31, 1924. Second injection, intratesticular, with 0.1 c.c. of abortin, March 8, 1924. Guinea-pig killed 38 days later. Grossly, this testicle was much reduced in size. Note the profound wandering cell infiltration with proliferation of the cells of Leydig, as well as the marked interstitial fibrosis. Note also the absence of all germinal elements and the extreme atrophy of the tubules. These are the typical late results of the abortin reaction.

left axilla with 1 c.c. Smears made from the injected material disclosed a rather heavy inoculation. The organism was grown on veal infusion agar, the plugs of the tubes having been dipped in paraffin. The method of growing the organism suggested by Fabyan,⁹ i. e., in

⁹ Jour. Med. Res., 1912, 26, p. 441.

connection with *B. subtilis*, was also attempted, but it was found that a much more abundant growth was obtained merely by the use of the plugs dipped in paraffin.

LESIONS OF *B. ABORTUS* INFECTION IN THE GUINEA-PIG

In the course of the infection, the following lesions were noted. The external appearance of the body was normal in the majority of cases; however, in one of the pigs a large abscess developed on the anterior abdominal wall, the body was extremely emaciated, the guinea-pig was very weak, and his hind quarters dragged when he attempted to move at any speed. This same pig exhibited an opacity of the cornea in the left eye. Another one of the pigs showed opaque cornea in both eyes.

The lymph nodes were enlarged in practically every case, but the distribution was not uniform. In those pigs inoculated in the axilla a distinctly enlarged, indurated lymph node was palpable. The retro-peritoneal nodes were always conspicuous. The spleen showed great enlargement as a rule, and directly under its capsule numerous minute nodules could be seen; these caused elevations on the surface, so that the spleen presented a finely granular exterior. The spleen was not always enlarged, however, for in some pigs which showed extensive nodules in the lung and elsewhere in the body, the spleen was of normal size. Not infrequently a fibrinous exudate covered the spleen.

The liver as a rule did not show extensive change. In two of the guinea-pigs there were a few grayish, translucent, pin point foci just visible in the substance under the capsule. The testicles showed changes in a great number of the pigs. In those that were inoculated intra-peritoneally, dense fibrous adhesions were formed between the visceral and parietal tunica vaginalis. In the majority of cases, nodules were found in the epididymis, which itself was distinctly enlarged. Even with the axillary infections, the epididymis contained nodules, and in one instance the body of the testicle, although the testicles in these cases were not adherent in the sac.

Lung lesions were noticed in guinea-pigs with a fairly well advanced infection. The nodules ranged from pin point to 1 mm. in diameter. The cut surface of the lung was nearly normal as a whole, with the exception of the few firm, irregular grayish areas. The pleura was not involved. A curious condition was observed in the ribs of the animals. There were distinct nodules, similar to a type of lesion discussed by Fabyan, on three of the ribs at the costochondral junction of one of

the guinea-pigs. This was in a pig which had been infected in the axilla. In those pigs which were infected intraperitoneally there developed easily torn fibrous adhesions between the spleen, left kidney and stomach, the liver and the diaphragm, and occasionally between loops of the intestine. These lesions did not occur in those pigs which were infected in the axilla.

Lesions in the lungs, spleen and lymph nodes, as well as those in the testicle and epididymis, were examined microscopically. The nodules in the lung were scattered throughout the entire organ, and consisted of accumulations of lymphoid cells and groups of epithelioid cells. The nodules were just beneath the pleura or about the blood vessels or bronchi. The interstitial tissue was extensively involved with small nodules. The structure of the nodules is similar to, if not identical with, the tuberculous nodule, namely, a central portion of epithelioid cells surrounded by the lymphoid cells.

The spleen was found to contain greatly dilated blood sinuses, and there was marked hyperplasia of the endothelial cells in and around the malpighian corpuscles, with an active cellular proliferation of the splenic pulp. The epithelioid cells, as in the lung, were observed in groups chiefly in relation to the malpighian bodies and directly beneath the capsule. Polymorphonuclear leukocytes were numerous. An occasional giant cell could be distinguished. The lymph nodes in the chronic cases were slightly enlarged and firm. This was evidently due to the infiltration with epithelioid cells. These groups of cells were present in such great numbers that the original lymphoid elements were pushed to the periphery of the node, where they appeared to be unaltered. Connective tissue hyperplasia was fairly pronounced, various groups of epithelioid cells being surrounded by a connective tissue stroma. No necrosis was observed.

PREPARATIONS OF ABORTIN

The abortin used for the skin and intratesticular test was prepared by two different procedures. First, I followed a technic similar to that used in the preparation of Koch's old tuberculin, namely, filtration of a 4 week culture of *B. abortus* through a Berkefeld filter and concentration of the filtrate on the water bath to about one-tenth of the original volume. This substance was then stored in an ice chest. A guinea-pig which had received a subcutaneous infection was given an intradermal skin test with this material. The reaction was negative at

the end of 24 hours. It may have been due to the fact that the infection had not made enough progress to cause a sensitization of the skin. However, it seemed best to discard this material.

Another method was then employed: A layer of chloroform, about 0.5 cm. thick, was introduced into an 8-day broth culture of the organism, and the flask was then stoppered and sealed with paraffin. This was shaken from time to time for a period of approximately 15 days. At the end of that time, the autolyzed product was filtered, placed over a small flame to free the material of chloroform, and eventually evaporated to about one half of the original volume. This material was tested on pigs infected with *B. abortus*, by means of the intradermal skin reaction, and it was found to give a strong reaction at the end of 24 hours. It was used in the experiments henceforth for both intradermal and intratesticular reaction.

RESULTS OF INJECTION OF ABORTIN

When abortin is injected into the testicle of a guinea-pig with a progressively active infection of *B. abortus*, a prompt reaction occurs, which, as a rule, reaches its maximum in from 24 to 36 hours. A guinea-pig killed within this period shows the injected testicle hyperemic and swollen. Microscopically, there is marked interstitial edema and epithelioid and wandering cell infiltration. The germ cells show a progressive degeneration, although an active spermatogenesis may still be noted within that short period. When the guinea-pig is allowed to live one month after its acute reaction, a profound degeneration of the testicle is observed. The testicle is decreased in size. The tubules are much smaller than normal, lined by undifferentiated epithelial cells with much cellular debris in the lumen; only occasional primary spermatocytes are present. A marked interstitial infiltration of wandering cells occurs. In many cases, as pointed out above, this picture is complicated by the presence of actual nodules produced by *B. abortus*.

These changes are shown in the accompanying illustrations. As with negligible exceptions, the findings in all the guinea-pigs examined conformed to the foregoing description, in order to conserve space the actual protocols for these animals will be omitted.

The results of the experiment demonstrate anew the similarity of the abortin reaction to the tuberculin reaction. The testicle of the guinea-pig infected with *B. abortus* (*melitensis*) is affected by abortin almost exactly as the testicle of the tuberculous guinea-pig is affected by

tuberculin. It may be added that the testicle of a tuberculous guinea-pig does not show any reaction when injected with abortin.

The results of the tests, however, are rather obscured by the fact that the testicle is prone to become infected in the course of development of *B. abortus* lesions. This is true with both routes of infection, intraperitoneal and subcutaneous; although the infection does not seem quite so severe with the subcutaneous infection, nodules developed in the testicle when they could not be found elsewhere in the body.

SUMMARY

In the testicle of a guinea-pig with *B. abortus* infection, an abortin reaction can be obtained which is in all respects similar to the tuberculin reaction in the testicle of the tuberculous guinea-pig. An acute response follows the introduction of abortin in the testicle of the abortus infected guinea-pig, reaching its maximum in 24-48 hours. The testicle becomes hyperemic and much swollen. Microscopically, intertubular edema and wandering cell infiltration are observed, with profound degeneration of the germ cells. In animals which are allowed to survive the acute response for several weeks, marked atrophy of the testicle with complete sterilization takes place.

These findings are complicated by the fact that *B. abortus* or *melitensis* infection is especially selective in producing lesions in the testicle regardless of the route of infection, whether intraperitoneal or subcutaneous, in marked contrast with tuberculosis in guinea-pigs.

The nodules produced by *B. abortus* resemble very closely those of tuberculosis, consisting of centrally grouped epithelioid cells surrounded by lymphocytes. Polymorphonuclear cells, are, however, more numerous.

As previously pointed out, the guinea-pig testicle is an excellent tissue to demonstrate the hypersensitiveness of infection.

A STUDY OF THE AGGLUTINATION TEST FOR BOVINE INFECTIOUS ABORTION

FRANK P. MATHEWS

From the Department of Veterinary Science, Purdue University Agricultural Experiment Station, Lafayette, Indiana

The presence of normal agglutinins for *B. abortus* (alcaligenes) in cattle serum has been the theme of much discussion. The following dilutions have been considered normal agglutinating limits: McFadyean and Stockman,¹ 1:25; Zwick,² 1:100; Smiley, Little, and Florence,³ 1:49; and Detre and Rohnyi,⁴ 1:50. Although the latter investigators were dealing with a badly infected herd, and 24% of all animals tested failed to give a reaction in dilutions as high or higher than 1:50, they accept this as the normal agglutinating limit.

The question naturally arises, Why should such a variety of opinions exist? H. R. Seddon⁵ concluded that the actual amount of serum used, and not the dilution, was the important factor in the agglutination test. However, his results can be explained by the ratio that exists between the serum and organisms when the titer of a serum is reached. The literature on the agglutination test for infectious abortion reveals one striking variation in technic, namely, the methods employed to determine the number of organisms in the antigen. For instance, an investigator may compare the turbidity of his antigen with a "paratyphoid bouillon density," or with a "48-hour typhoid bouillon culture." McFarland's nephelometer may have been used, but frequently the methods of standardization have not been discussed.

Since there is disagreement as to the interpretation of a reaction, and variation in technic exists, it was decided to determine the importance of the following factors: (1) the number of organisms in the antigen; (2) the period of incubation; and (3) the hydrogen-ion concentration.

In the main, the methods used to determine the number of organisms in an antigen are based on the turbidity that organisms impart to the solution. As a standard for comparison, the method giving the most constant results is probably McFarland's nephelometer,⁶ and for this reason it was chosen as a standard.

Received for publication, July 3, 1924.

¹ Jour. Comp. Path. & Therap., 1907, 20, p. 22.

² Deutsch. Tier. Wchnschr., 1911, 19, p. 781.

³ Jour. Exper. Med., 1919, 30, p. 341.

⁴ Berl. Tier. Wchnschr., 1922, 38, p. 345.

⁵ Jour. Comp. Path. & Therap., 1915, 28, p. 20.

⁶ Jour. Am. Med. Assn., 1907, 49, p. 1176.

An antigen was prepared by growing 3 different cultures of *Alcaligines abortus* from widely separated sources on agar flats for 48 hours, and washing off the growth with phenolized salt solution (NaCl 0.85%, phenol 0.5%). This antigen was diluted to correspond to a turbidity of 3 with McFarland's nephelometer (3 c.c. of 1% BaCl₂ and 97 c.c. 1% con. H₂SO₄). This was designated as antigen 1. A definite amount of this antigen was diluted with an equal amount of phenolized salt solution and designated antigen 2. A definite amount of antigen 2 was diluted with an equal amount of phenolized salt solution and designated as antigen 3. The 3 antigens were tested with the serums of 170 cows from 9 herds. The titer of each serum for each antigen was determined as soon as possible after the blood was drawn. Two readings were made in all tests, the first after 18, and the second after 42 hours' incubation.

For convenience the results have been divided into 3 groups. Group 1 was composed of serum from 82 animals, all of which gave negative results with antigen 1. Fifty-three of the animals gave negative results with antigen 2, and 21 gave negative results with antigen 3. Twenty-six or 31% had a titer of 1:25 with antigen 2, and 61, or 74% had a titer of 1:25, and 12, or 15%, a titer of 1:50 with antigen 3. From these results it may be concluded that the turbidity of an antigen is an important factor to keep in mind when considering the limits of normal agglutinins.

Group 2 was composed of the serum from 8 individuals, all of which gave the same results on the 18-hour reading, and for this reason only the chart of one animal is given. This group is of interest because the reactions fall on the border line between specific agglutination and normal agglutinating limits.

REACTIONS OF SERUM 1, GROUP 2

Antigens	Dilution					Hours
	1:25	1:50	1:100	1:200	1:500	
1	+	0	0	0	0	18
	+	0	0	0	0	42
2	+	+	0	0	0	18
	+	+	..	0	0	42
3	+	+	+	0	0	18
	+	+	+	±	0	42

+, Complete agglutination; ±, partial agglutination; 0, no agglutination.

A history of this group is of interest. The serum that gave the results is from an animal that had never aborted, although *B. abortus* was isolated from the normally discharged fetal membranes. Two other cows in the group delivered live calves but retained their placentas, and *A. abortus* was isolated from the fetal membranes in both cases. All 3 gave positive reactions with the complement-fixation test. The remaining 5 were animals in badly infected herds and gave positive reactions with the complement-fixation test. A study of the 18-hour reading on the chart reveals two outstanding facts: first, the ratio that exists between serum and organisms, when the titer of a serum is reached. The titer of the serum with antigen 1 was 1:25. The titer of the same serum against antigen 2 was 1:50; and against antigen 3 was 1:100, thus giving 3 titers for the same serum, but the ratio of serum to organisms in all three cases was the same since antigen 2 contained only one-half as many organisms as antigen 1, and antigen 3, one-half as many organisms as antigen 2. Second: As the turbidity of an antigen decreases, its agglutinability increases.

Group 3 was composed of the serums from the 80 remaining animals, all of which gave higher reactions than the preceding group. No one chart could be given as an illustration, as a few gave reactions just higher than the preceding, while a number possessed very high titers. The remainder fell between these two extremes. This group also emphasized the fact that a definite ratio exists between serum and organisms when the titer of a serum is reached, and as the turbidity of an antigen decreases its agglutinability increases. This increased agglutinability was more pronounced with some serums than with others. When the turbidity of an antigen was decreased one-half, its agglutinability was doubled in 65% of all positive reactions. However, in 30% of the positive reactions, the agglutinability was more than doubled, i. e., the same serum that had a titer of 1:500 dilutions with antigen 1, had a titer of 1:2,500 with antigen 2; and one of 1:7,500 with antigen 3. Another serum had a titer of 1:50 dilution with antigen 1; 1:200 with antigen 2, and 1:1,000 with antigen 3. These reactions emphasize the importance of turbidity of antigen in plotting a reaction curve, especially if a new antigen is to be used before the curve is completed. Five per cent. of the positively reacting serums gave somewhat atypical results as regards the influence of turbidity of antigen, i. e., a serum had a titer of 1:200 dilution with all 3 antigens. Another had a titer of 1:1,000 with antigens 1 and 2, and 1:2,000 with antigen 3.

The results obtained on the 42-hour readings were inconstant with antigens 2 and 3. Antigen 1, however, was constant, giving an increased reaction in only one test. By increased reaction is meant complete or partial agglutination in a higher dilution than was found on the 18-hour reading. With antigen 2, 28, or 16.4%, of all tests gave an increased reaction. With antigen 3, 66, or 30.8%, showed an increased reaction. In all, 94, or 47.2%, of the tests showed increased reaction, but of these, 44.6% were confined to group 1, or the negative reactions. This may be considered as evidence that the reaction after the first 18 hours' incubation is probably due to something other than specific agglutinins.

In these herds, the average titer of positively reacting serums was found to be higher in herds in which abortion was of long duration than in herds in which the disease was more of an explosive nature and of shorter duration.

Three antigens having a hydrogen-ion concentration of 10^{-6} , 10^{-7} and 10^{-8} were prepared and tested against 20 serums ranging in titer of 1:25 to 1:2,000 dilution. No variation as to agglutinability was noticed, and the work was discontinued.

SUMMARY

Normal agglutinins are influenced to a considerable extent by the turbidity of an antigen.

A definite ratio exists between the amount of serum and the number of organisms in the antigen when the titer of a serum is reached. As the number of organisms in an antigen decreases, its agglutinability increases, thus emphasizing the importance of turbidity of antigen in plotting a reaction curve.

Eighteen hours' incubation gave the most satisfactory results. This work indicates that agglutination after 18 hours is probably not due to specific agglutinins.

Hydrogen-ion concentration between the limits of 10^{-6} and 10^{-8} has no influence on the agglutination test for infectious abortion.

RECOVERY OF BACILLUS TERTIUS FROM STOOLS OF INFANTS

IVAN C. HALL AND KIYOSHI MATSUMURA

From the University of California, Berkeley, and the Japanese Maternity Home, San Francisco

We have recently studied a series of 8 stools from young Japanese children, with special reference to their content of sporulating anaerobes. Feces were collected on clean, ironed diapers. A small piece the size of a pea was inoculated from each into freshly boiled deep brain medium. This was again heated to 80 C. for 20-30 minutes, to exclude non-sporulating micro-organisms, and incubated at 37 C. In cases in which aerobic spore-formers appeared, they were eliminated by selective bacteriostasis.¹ Pure cultures were isolated by the deep agar method and identified by one of us (M) and confirmed by the other (H), with the following results:

TABLE 1
ISOLATION OF CULTURES

Number	Age	Diet	Condition	Result
1	4 months	Bottle	Intestinal irregularity, colic, tenesmus	<i>B. multifementans</i>
2	12 hours	Normal	No spores
3	18 months	Breast and bottle	Normal	<i>B. welchii</i>
4	6 months	Bottle	Normal	<i>B. tertius</i>
5	8 months	Bottle	Chronic gastro-enteritis, eczema	<i>B. tertius</i>
6	5 months	Bottle	Fever, acute gastro- enteritis,* eczema	No anaerobic spores
7	6 days	Breast	Normal	<i>B. tertius</i>
8	10 days	Breast	Normal	<i>B. tertius</i>
	4 months	Breast and bottle	Normal	<i>B. welchii</i>

* Specimen secured after eatharsis.

Two of the specimens gave no evidence of anaerobic spores; one contained only aerobic spores.

B. tertius spores were recovered 4 times from 3 of these patients. One, a normal breast-fed baby, was 6 days old at the time of the first collection and still harbored the organism at 10 days of age; the other 2 were 6 and 8 months old, respectively, and bottle fed. Of the latter 2, one was normal; the other suffered from eczema and chronic gastro-enteritis. Bacteria resembling *B. tertius* morphologically were also seen

Received for publication, Aug. 1, 1924.

¹ Hall: Jour. Am. Med. Assn., 1919, 72, p. 274; Jour. Infect. Dis., 1920, 27, p. 576.

in specimens from 2 other children, 4 and 18 months old, respectively, but were lost before isolation. *B. tertius* has been isolated in this laboratory also from the feces of a normal man and of a 10-year-old healthy girl and from the feces of a sheep.

B. welchii also was isolated from the stools of 2 of the infants, and *B. multifermantans*² from 1.

B. tertius was named as the third most frequent organism recovered from war wounds by Henry.³ It is characterized by its elongate terminal spores, its lack of proteolytic or even gelatinolytic power, and its rather marked saccharolytic properties.

An additional point in the bacteriology, overlooked by previous observers, is that *B. tertius*, like *B. histolyticus*,⁴ grows aerobically in delicate but distinct discrete colonies on the surface of blood-agar and plain meat-infusion-agar slants, but usually not on commercial meat-extract-agar slants. Also like *B. histolyticus*, it sporulates less freely under aerobic conditions. Whether it will maintain its characters through long repeated aerobic cultivation, remains to be tested; it will at least maintain them through several aerobic transfers. These observations are based not only on the strains mentioned above, but also on a strain (Renard—from a case of gas gangrene in 1918) from the National Collection of Type Cultures of the Lister Institute at Chelsea Gardens, London, on a previously unidentified strain isolated by Prof. J. P. Scott, of the University of Kansas, at Manhattan, from bovine blackleg, and on a strain received from Prof. Camillo Ninni, of the University of Naples, Italy, under the name, "*B. spermoides*."

This last deserves special mention. Ninni⁵ described a new "anaerobic bacillus of the soil," which he named *B. spermoides* because of a striking resemblance of the terminally spored rods to spermatozoa. It was isolated from the earth of a high mountain (Scaglioso), "certainly inaccessible to mammals," in an effort to throw light on the ancient telluric-fecal controversy over the natural habitat of *B. tetani*. Ninni described and pictured an organism corresponding in every way with *B. tertius* except for gelatin liquefaction. But the strain sent by him fails to liquefy gelatin, and is, in fact, none other than *B. tertius*.

Henry noted that an organism similar to *B. tertius* was recovered by Rodella⁶ in 1902 from the stools of nursing infants, but Rodella's

² Stoddard: *Lancet*, 1919, 196, p. 12.

³ *Jour. Path. & Bacteriol.*, 1917, 21, p. 344.

⁴ Hall: *Proc. Soc. Exper. Biol. & Med.*, 1923, 20, p. 501.

⁵ *Pathologica*, 1920, 12, p. 385.

⁶ *Ztschr. f. Hyg. und Infektionskr.*, 1902, 39, p. 201.

bacillus was claimed to be pathogenic for laboratory animals, while *B. tertius* never is, at least in pure experimental inoculations.

The possible identity of *B. gazogenes*, recovered by Choukevitch⁷ in 1911 from the large intestine of the horse, should also be investigated fully; positive proof would involve priority of the name used by him for this species.

Unnamed anaerobes with elongate terminal spores have been observed frequently in wound infections; as Henry noted, von Hibler's⁸ "Bacillus IX" and Fleming's⁹ "Bacillus Y" were evidently related to, if not identical with, *B. tertius*. Robertson¹⁰ recovered "Hibler IX" from a wound and gave a good description, which equally fits *B. tertius*, and the description of a "bacillus resembling *B. tetani*" by Adamson and Cutler¹¹ seems to correspond in respect to cultural features, if not exactly in respect to morphology. But Goadby^{12 13} evidently confused *B. putrificus*, or some other putrefactive round-spored organism, with von Hibler's "Bacillus IX."

More recently, *B. tertius* has been recovered from wound infections by Weinberg and Seguin¹⁴ and by Adamson,¹⁵ from human feces by Kendall, Day, and Walker¹⁶ and from ground beef by Hoffstadt.¹⁷

SUMMARY

Our findings suggest that *B. tertius* is an early and frequent invader of the intestinal tract of infants, both well and unwell. It occurs also in the feces of older children and adults, and in the feces of sheep. There is no evidence that it has any direct pathologic significance. Like *B. welchii* and *Bact. coli*, *B. tertius* is actively lactolytic, but it is less putrefactive than either, notwithstanding the premature opinions of Goadby and Fleming to the contrary, which were and are unsupported by pure cultural evidence. It is significant that no putrefactive bacteria were encountered in any of our cultures and that the anaerobic organisms found should be among the most actively fermentive known.

B. tertius is facultatively, not obligately, anaerobic.

⁷ Ann. de l'Inst. Pasteur, 1911, 25, pp. 247, 345.

⁸ Untersuchungen über die pathogenen Anaëroben, Fisher, 1908.

⁹ Lancet, 1915, 2, pp. 376, 628.

¹⁰ Jour. Path. & Bacteriol., 1916, 20, p. 327.

¹¹ Lancet, 1917, 1, p. 688.

¹² Ibid., 1916, 2, pp. 89, 585 and 851.

¹³ Jour. Roy. Microsc. Soc., 1917, 1, p. 269.

¹⁴ La Gangrène gazeuse, 1917.

¹⁵ Jour. Path. & Bacteriol., 1919, 22, p. 345.

¹⁶ Jour. Infect. Dis., 1922, 30, p. 141.

¹⁷ Am. Jour. Hyg., 1924, 4, p. 43.

A STUDY OF BACTERIOPHAGE WITH ANTIBACTERIOPHAGIC SERUM

LLOYD ARNOLD AND EMIL WEISS

*From the Department of Bacteriology, Pathology and Preventive Medicine, Loyola University
School of Medicine, Chicago*

Bordet and Cuica¹ were the first to record the presence of an antibacteriophagic substance in the serum of an animal immunized with bacteriophage. The addition of this antiserum to a culture of bacteria and bacteriophage would prevent lysis, and subcultures from this mixture gave regular and normal colony formation, i. e., there was no evidence of bacteriophagic activity on the subcultures. These workers concluded that the lytic activity of the bacteriophage had been neutralized by the antilysins of the immune serum. D'Herelle and Eliava² repeated this experiment and found that a mixture of antibacteriophagic serum and bacteriophage would prevent lysis of a susceptible bacterium, but when subcultures were made from this growth, lysogenic or bacteriophagic activity was manifested after a certain time. They concluded that antilysins had only inhibited the lysins of the bacteriophage for the time being and that neutralization did not take place. We wish to record in this paper the results of our experiments dealing with the question whether the antilysins inhibit or neutralize the lysins of the bacteriophage and whether the antilytic serum acts on the bacterium and changes its susceptibility. We were also interested in passively immunizing a susceptible bacterium against the lytic activity of a bacteriophage.

TECHNIC

Bacteriophage that was active for various members of the colityphoid group was used. The bacteriophagic suspensions were prepared both by D'Herelle's and the bottom layer agar method.³ The relative strength of the bacteriophage was determined in the following way: A series of agar plates were seeded with one loop of a 24-hour old broth culture of the respective bacterium; decreasing amounts of the bacteriophagic suspension were added, and the surface smeared well with a bent glass spreader and incubated for 24 hours. A strong, active

Received for publication, June 19, 1924.

¹ Compt. rend. Soc. de biol., 1924, 84, p. 28.

² Ibid., p. 719.

³ Arnold: Jour. Lab. & Clin. Med., 1923, 8, p. 720.

or potent bacteriophage was one that in a dose of 0.05 c c. or less would produce almost total inhibition of growth, with practically all of the visible colonies irregular in outline. This amount of bacteriophage was taken as our "unit" dose. This method gives more reliable results than the addition of bacteriophage to a broth culture and counting the "holes" or "plaques" when subcultured on agar. This standard of evaluation of the potency of the bacteriophage was discussed in a previous publi-

TABLE 1
A. GENERAL TITRATION OF BACTERIOPHAGE

No. of Plates	Bacteriophage	Bacteria	Results on Plates (24 Hrs.)
1	0.25	1 loop	Sterile = 100% bacteriophage activity = 32 units
2	0.2	1 loop	Sterile = 100% bacteriophage activity = 16 units
3	0.15	1 loop	Sterile = 100% bacteriophage activity = 8 units
4	0.1	1 loop	Sterile = 100% bacteriophage activity = 4 units
5	0.05	1 loop	Sterile = 100% bacteriophage activity = 2 units
6	0.025	1 loop	Sterile = 100% bacteriophage activity = 1 unit
7	0.0125	1 loop	= 50% bacteriophage activity = $\frac{1}{2}$ unit
8	0.00625	1 loop	= 25% bacteriophage activity = $\frac{1}{4}$ unit
9	0.003125	1 loop	= 12.5% bacteriophage activity = $\frac{1}{8}$ unit
10	0.0015625	1 loop	= 6.25% bacteriophage activity = $\frac{1}{16}$ unit

B. SPECIAL TITRATION OF BACTERIOPHAGE

11	1 unit (0.025 c c.)	1 loop	Sterile..... = 100% bacteriophage activity
12	$\frac{9}{10}$ (0.025 c c.)	1 loop	Gradually decreasing irregularity = 90% bacteriophage activity
13	$\frac{8}{10}$ (0.025 c c.)	1 loop	Gradually decreasing irregularity = 80% bacteriophage activity
14	$\frac{7}{10}$ (0.025 c c.)	1 loop	Gradually decreasing irregularity = 70% bacteriophage activity
15	$\frac{6}{10}$ (0.025 c c.)	1 loop	Gradually decreasing irregularity = 60% bacteriophage activity
16	$\frac{5}{10}$ (0.025 c c.)	1 loop	Gradually decreasing irregularity = 50% bacteriophage activity
17	$\frac{4}{10}$ (0.025 c c.)	1 loop	Gradually decreasing irregularity = 40% bacteriophage activity
18	$\frac{3}{10}$ (0.025 c c.)	1 loop	Gradually decreasing irregularity = 30% bacteriophage activity
19	$\frac{2}{10}$ (0.025 c c.)	1 loop	Gradually decreasing irregularity = 20% bacteriophage activity
20	$\frac{1}{10}$ (0.025 c c.)	1 loop	Gradually decreasing irregularity = 10% bacteriophage activity

cation.³ Table 1, A shows the results in a typical titration of a bacteriophage, the amount of the lytic substance, the dose of bacteria with the results expressed in terms of bacteriophagic activity and unit values. Table 1, B shows the decreasing percentage of lysogenic colonies on an agar plate, seeded with progressively smaller amounts of bacteriophage, but the same dose of bacteria in each instance. Bacteriophage kept at room or icebox temperature does not deteriorate or change in its potency, our longest observation being 10 or 12 months.

Our method of preparation of the antibacteriophagic or antilytic serum has already been described.⁴ We determined as antilytic unit the smallest amount of antilytic serum that would cause normal or

TABLE 2
A. GENERAL TITRATION OF ANTILYSIN

No. of Plates	Antilytic Serum	Bacteriophage	Bacteria	Results on Plates (24 Hrs.)		
1	0.25 c c.	1 unit	1 loop	Normal growth = 0 %	bacteriophage = 100 %	antilytic = 64 units activity
2	0.2	1 unit	1 loop	Normal growth = 0 %	bacteriophage = 100 %	antilytic = 32 units activity
3	0.15	1 unit	1 loop	Normal growth = 0 %	bacteriophage = 100 %	antilytic = 16 units activity
4	0.1	1 unit	1 loop	Normal growth = 0 %	bacteriophage = 100 %	antilytic = 8 units activity
5	0.05	1 unit	1 loop	Normal growth = 0 %	bacteriophage = 100 %	antilytic = 4 units activity
6	0.025	1 unit	1 loop	Normal growth = 0 %	bacteriophage = 100 %	antilytic = 2 units activity
7	0.0125	1 unit	1 loop	Normal growth = 0 %	bacteriophage = 100 %	antilytic = 1 unit activity
8	0.00625	1 unit	1 loop	= 50 %	bacteriophage = 50 %	antilytic = ½ unit activity
9	0.003124	1 unit	1 loop	= 75 %	bacteriophage = 25 %	antilytic = ¼ unit activity
10	0.0015625	1 unit	1 loop	= 87.5 %	bacteriophage = 12.5 %	antilytic = ⅛ unit activity

B. SPECIAL TITRATION OF ANTILYSIN

11	1 unit (0.0125 c c.)	1 unit	1 loop	Normal growth = 0 %	bacteriophage = 100 %	antilytic activity
12	$\frac{9}{10}$ (0.0125 c c.)	1 unit	1 loop	Gradually increasing irregularity = 10 %	bacteriophage = 90 %	antilytic activity
13	$\frac{8}{10}$ (0.0125 c c.)	1 unit	1 loop	Gradually increasing irregularity = 20 %	bacteriophage = 80 %	antilytic activity
14	$\frac{7}{10}$ (0.0125 c c.)	1 unit	1 loop	Gradually increasing irregularity = 30 %	bacteriophage = 70 %	antilytic activity
15	$\frac{6}{10}$ (0.0125 c c.)	1 unit	1 loop	Gradually increasing irregularity = 40 %	bacteriophage = 60 %	antilytic activity
16	$\frac{5}{10}$ (0.0125 c c.)	1 unit	1 loop	Gradually increasing irregularity = 50 %	bacteriophage = 50 %	antilytic activity
17	$\frac{4}{10}$ (0.0125 c c.)	1 unit	1 loop	Gradually increasing irregularity = 60 %	bacteriophage = 40 %	antilytic activity
18	$\frac{3}{10}$ (0.0125 c c.)	1 unit	1 loop	Gradually increasing irregularity = 70 %	bacteriophage = 30 %	antilytic activity
19	$\frac{2}{10}$ (0.0125 c c.)	1 unit	1 loop	Gradually increasing irregularity = 80 %	bacteriophage = 20 %	antilytic activity
20	$\frac{1}{10}$ (0.0125 c c.)	1 unit	1 loop	Gradually increasing irregularity = 90 %	bacteriophage = 10 %	antilytic activity

regular growth when added to the surface of an agar plate seeded with one loop of bacteria and one unit of bacteriophage. Table 2 illustrates the titration of an antibacteriophagic serum. The doses of both the

⁴ Weiss and Arnold: Jour. Infect. Dis., 1924, 34, p. 317.

bacteria and bacteriophage remain constant; the antilysins are progressively decreased in amount. The results are expressed in both lytic and antilytic activity. The second part of this table (B) shows the neutralizing effect of the antilysins.

Blood was taken from the ear vein of rabbits as it was needed. The antilytic titer usually remained fairly constant for the respective bacteria for 5 to 6 months after it had reached its maximum activity. When the titer dropped below 0.5 c.c. of undiluted serum, the rabbits were again immunized. In this instance, small doses of the antigen were repeated several times at short intervals, and finally gradually increased to the same amount as previously used for the initial immunization. This was necessary because of the high bacterial protein content of the bacteriophagic suspensions.

We determined the precipitin, agglutinin, opsonin and amboceptor content of the antisera as well as the antilysin content.

In all quantitative work with the bacteriophage, it is necessary to use Petri dishes of the same diameter and agar of the same thickness. This is a very important point, as will be shown in a subsequent paper dealing in detail with this and closely allied questions.

B. typhosus was used in all the experiments in this article.

THE INFLUENCE OF ANTILYTIC SERUM ON THE BACTERIA

A series of tubes was prepared of antilytic serum in varying concentrations in broth; the total volume in each tube was 2 c.c. Each tube was seeded with 0.1 c.c. of a 24-hour broth culture of the susceptible bacterium and incubated for 48 hours. Normal serum in the same dilutions and plain broth in the same volume were seeded in the same manner and incubated as controls. All tubes were then centrifuged, and the supernatant fluid transferred separately under sterile precautions to other tubes. The bacterial sediment was resuspended in both and centrifuged; this was repeated 4 times. Finally the bacteria were suspended in the same quantity of broth (2 c.c.) and tested in the usual manner on agar plates as to their respective resistance or susceptibility to the bacteriophagic substance. The bacteria that had been grown in the antilytic serum were just as susceptible to bacteriophagic activity as were those grown in the normal serum and plain broth.

The antilytic serum dilutions removed from the bacterial sediment after the first centrifugation were heated for 1 hour at 60 C.⁵ In a

⁵ This temperature kills the bacteria, but, as shown in table 3, only slightly decreases the potency of the antilysins.

duplicate experiment, the antilytic serum dilutions were passed through a Berkefeld filter. Fresh antilytic serum in the same dilutions was heated and filtered in the same way for controls. The antilytic content of these dilutions of antiserum was determined in the manner described. The titer was the same for the antilytic serum that had been incubated with the bacteria for 48 hours as for the antilytic serum freshly prepared. We could not find that the bacteria had removed or changed the antilytic content of the serum. The antilytic serum that had been in contact with the bacteria showed a decrease in agglutinins, opsonins and amboceptors. There was a decrease in antilysins from all the dilutions after passing through the Berkefeld filter. We found that this filter holds back antilysins in all the experiments attempted.

Unless the bacteria that have been suspended in the antilytic serum are washed well, there will be enough antilysins carried over to cause partial neutralization during the first few passages. A few observers have reported some temporary acquired resistance as a result of treatment of susceptible bacteria with antilytic serum; if the source of error mentioned in the foregoing is removed, we think there will be no evidence to support the theory of passive immunization of bacteria against lysis of bacteriophagic origin.

THE LYSIN-ANTILYSIN REACTION

Table 3 gives in condensed form the effect of temperature on the activity of the antilysins. The antiserum, undiluted and diluted, as shown in the first column, was heated for one hour at the temperatures indicated; then 0.05 c.c. of the bacteriophage and one loop of the bacteria added, and smeared well over the surface. The lysogenic

TABLE 3
THE INFLUENCE OF THE TEMPERATURE ON THE WEAKENING OF ANTILYSINS AND BACTERIOPHAGE

Antilysin	Bacteriophage	Bacteria	Results on Plates* after 1 Hour at						
			37 C.	50 C.	60 C.	70 C.	80 C.	90 C.	100 C.
Undil., 0.05 c.c.	0.05 c.c.	1 loop	0	0	10	100	100	100	100
1/10, 0.05 c.c.	0.05 c.c.	1 loop	10	20	20	60	60	90	100
1/20, 0.05 c.c.	0.05 c.c.	1 loop	20	20	20	65	65	90	100
1/30, 0.05 c.c.	0.05 c.c.	1 loop	40	40	40	85	85	100	100
1/40, 0.05 c.c.	0.05 c.c.	1 loop	60	60	60	85	90	100	100
1/50, 0.05 c.c.	0.05 c.c.	1 loop	80	80	85	95	95	100	100
1/100, 0.05 c.c.	0.05 c.c.	1 loop	80	85	90	95	95	100	100
—	0.05 c.c.	1 loop	100	25	10	5	0	0	0

* Bacteriolytic activity expressed in percentages.

activity is recorded in percentage as mentioned. The diluted antilynsins are not so sensitive to heat as the undiluted. The lower line of the table shows the effect of these temperatures on the bacteriophage alone. Antilynsins can be heated to 60-70 C. when diluted and still retain enough antilytic power to be useful for certain experimental work. The following explanation of a single line of this table may be of assistance in its proper interpretation. The first line shows that the undiluted anti-

TABLE 4
THE INFLUENCE OF TEMPERATURE ON THE LYSIN-ANTILYSIN-REACTION *

No. of Plates	Antilytic Serum Mixed with		Bacteriophage	Bacteria	Results on Plates
	Bacteriophage and Heated 1 Hour at 65 C	Broth			
1	0.05 c c. antilysin + 0.05 c c. bacteriophage	0.05 c c.	1 loop	15% irregularity
2	0.025 c c. antilysin + 0.05 c c. bacteriophage	0.05 c c.	1 loop	25% irregularity
3	0.01 c c. antilysin + 0.05 c c. bacteriophage	0.05 c c.	1 loop	60% irregularity
4	0.005 c c. antilysin + 0.05 c c. bacteriophage	0.05 c c.	1 loop	90% irregularity
5	0.05 c c. antilysin + 0.05 c c. broth	0.05 c c.	1 loop	Normal growth
6	0.025 c c. antilysin + 0.05 c c. broth	0.05 c c.	1 loop	Trace irregularity
7	0.01 c c. antilysin + 0.05 c c. broth	0.05 c c.	1 loop	25% irregularity
8	0.005 c c. antilysin + 0.05 c c. broth	0.05 c c.	1 loop	40% irregularity
9	0.05 c c.	1 loop	100% irregularity
10	Heated 1 Hour at 65 C.	1 loop	5% irregularity
11	1 loop	Normal growth

* Bacteriophage activity expressed in percentages.

serum was heated at the various temperatures and then mixed with fresh untreated bacteriophage and bacteria on the agar plate; at 37 and 50 C., the antilynsins were undamaged, i. e., protection against lysis was complete; at 60 C. 10% of the colonies were irregular or lysogenic; at 70 to 100 C., all antilytic activity was destroyed.

Table 4 gives in condensed form an experiment in which we mixed antilynsins with bacteriophage and heated for 1 hour at 65 C. (plates 1 to 4 inclusive). Antilynsins were mixed with broth in the same concentration and heated in the same manner (5 to 8 inclusive). These

heated mixtures were then tested as to their respective antilytic power against 1 unit of bacteriophage and a loop of susceptible bacteria. Plate 9 gives the bacteriophage and bacteria, 10 the heated bacteriophage and bacteria, 11 the bacteria only. It will be noticed that the heated antilysin-lysin mixture does not have the antilytic content of the heated antilysin-broth mixture, i. e., 1 unit of bacteriophage produces greater irregularity or bacteriophagic activity in the latter than in the former. If the antilysin-lysin reaction had been only inhibition, the results should have been about the same, because the control heated bacteriophage shows a loss of 95% of its lytic power at this temperature. We interpret this difference as being due to a neutralization in the antilysin-lysin mixture and not inhibition.

TABLE 5
THE INFLUENCE OF TIME AND TEMPERATURE ON THE LYSIN-ANTILYSIN-REACTION

Antilysin	Bacteriophage	Bacteria	Results with Mixtures of Bacteriophage and Antilysins after		
			48 Hours at 37 C.	48 Hours in Icebox	Immediately
0.03 c c.	0.05 c c.	1 loop 24 hrs. in broth	Normal growth	Normal growth	Normal growth
0.015 c c.	0.05 c c.	1 loop 24 hrs. in broth	Normal growth	Normal growth	Normal growth
0.006 c c.	0.05 c c.	1 loop 24 hrs. in broth	Normal growth	35% irreg.	70% irreg.
0.003 c c.	0.05 c c.	1 loop 24 hrs. in broth	60% irreg.	65% irreg.	80% irreg.
0.0015 c c.	0.05 c c.	1 loop 24 hrs. in broth	70% irreg.	80% irreg.	90% irreg.
0.00075 c c.	0.05 c c.	1 loop 24 hrs. in broth	75% irreg.	90% irreg.	95% irreg.
—*	0.05 c c.	1 loop 24 hrs. in broth	95% irreg.	95% irreg.	95% irreg.

* Bacteriophage controls.

Table 5 shows an experiment illustrating the influence of time and temperature on the lysin-antilysin reaction. It will be seen that the experiment was made in triplicate; one series of lysin-antilysin was mixed and kept for 48 hours at 37 C., one series 48 hours in the icebox (2-4 C.), then the same amount of each was added to the surface of agar plates after this 48-hour period plus a loop of a 24-hour culture of the bacteria. In the other series, the mixture was added immediately, after being put together, on the surface of the agar plate and seeded in the same way with bacteria. All plates were incubated for 24 hours. Several controls without antilysins were made at the same time, and the lower line of the table shows the results obtained with such a control. It will be seen that the lytic activity was greatest in the

mixture that was immediately added to the plates and least in the mixture that was incubated for 48 hours at 37 C., the icebox series occupying an intermediate position. Time and temperature influence this reaction as they do the toxin-antitoxin reaction. We interpret this as a neutralization process, similar to the toxin-antitoxin neutralization.

A series of mixtures of a certain quantity of bacteriophage and antiserum was passed through the Berkefeld filter after standing together for certain periods of time and the filtrates tested in the usual manner for lytic activity. Table 6 gives in detail the quantities used and the time intervals, with the results.

This was a repetition of Martin and Cherry's⁶ experiment with snake venom and its specific antitoxin. Our results are the same as theirs, that is, the time factor plays a part in the lysin-antilysin union, and the antilysin molecule does not pass the filter as readily as the lysin molecule.

TABLE 6
THE INFLUENCE OF TIME ON THE LYSIN-ANTILYSIN REACTION

Bacteriophage	Antilysin	Bacteria	Results of Mixtures of Bacteriophage and Antilysins Filtered				
			Immedi- ately	After 3 Hours	After 6 Hours	After 12 Hours	After 24 Hours
0.05 c.c.	0.05 c.c.	1 loop	30%	10%	Trace irreg.	Normal growth	Normal growth

Danysz⁷ and later Bordet⁸ found that when a toxin and its homologous antibody were brought into contact with each other the degree of neutralization depended on the manner in which the 2 were put together. When the toxin was added to the antitoxin in 2 fractions, a considerable length of time intervening between the additions, the final mixture was much more toxic than when the total amount was added at once. As our lysin-antilysin reaction paralleled that of the toxin-antitoxin reactions, we were interested in finding out whether it would also exhibit this so-called "Bordet-Danysz phenomenon" when mixed in a similar manner. Table 7 is a record of the average of results of such an experiment. When the lysins are added in 2 fractions, 24 hours intervening between the 2, the protection is not so great, i. e., the mixture is more "toxic" for the bacteria than when the total amount of lysins are added at one time. We have found that the larger the

⁶ Proc. Roy. Soc., 1898, 63, p. 420.

⁷ Ann. de l'Inst. Pasteur, 1902, 16, p. 33.

⁸ Ibid., 1903, 17, p. 161.

second fraction and the longer the time interval between the 2 fractions, the more marked is the toxicity in the final mixture. If the antilyns are added to the lysins in the same fractional manner, the same phenomenon occurs.

Tables 2, 3, 4 and 5 show that the lysin-antilysin reaction follows the laws of definite proportion. This corresponds to the toxin-antitoxin reaction or neutralization. The method of determining bacteriophagic activity by adding the lytic principle to fluid cultures of the bacteria and using the degree or time of lysis, that is, the clearing of the visible bacterial growth, is not so delicate as the agar plate method⁹ we have used.^{3, 4} Small amounts of bacteriophage not detectable by the broth tube method are easily demonstrated by the agar plate method.

TABLE 7
DANYSZ-BORDET PHENOMENON

No. of Plates	Mixtures of, at 37 C. for 24 Hours			After 24 Hours at 37 C. Added			
	Bacterio- phage	Anti- lysin	Broth	Bacterio- phage	Anti- lysin	Bac- teria	Results on Plates
1	0.1 c c.	0.05 c c.	1 loop	20% irregularity
2	0.05 c c.	0.05 c c.	0.05 c c.	1 loop	35% irregularity
3	0.1 c c.	0.05 c c.	1 loop	95% irregularity
4	0.05 c c.	0.05 c c.	0.05 c c.	1 loop	95% irregularity
5	0.05 c c.	0.05 c c.	1 loop	Normal growth
6	0.05 c c.	0.025 c c.	0.025 c c.	1 loop	10% irregularity
7	0.05 c c.	1 loop	100% irregularity
8	1 loop	Normal growth

Bacteriolytic activity expressed in percentage.

A fixed amount of bacteriophage was placed in a series of tubes and decreasing amounts of antilyns added. In this experiment, there was purposely an excess of antilyns added to the first tube; the last tube received a small amount of antilyns, too small to exert a detectable neutralizing effect. Three drops from each tube were placed on the surface of an agar plate with susceptible bacteria and smeared well with a sterile bent glass spreader. After 24 hours' incubation, the agar was removed separately from each plate and put in small flasks with 10 c c. of distilled water, placed in the shaking machine over night, and then passed through a Berkefeld filter. These filtrates were each tested separately as to their lytic and antilytic power. It was found that the filtrate from the plate that contained the minimal amount of antilyns that showed regular and normal growth of the bacteria (the neutralizing dose) did not contain lysins or antilyns, even after repeated

⁹ We are now engaged in a detailed quantitative study of this question.

passage of bacteria. The filtrates from the plates with larger amounts of antiserum showed antilyns in the filtrates, and the amount of antilytic activity was comparable to the amount of antiserum excess added. The filtrates from the plates showing irregular or lysogenic growths, that is, those receiving smaller amounts of antilyns than was necessary to neutralize the lysins present, contained bacteriophage or lysins in quantities to some extent comparable to the excess of lysins present on the original agar plate.

Bordet worked with an antiserum having a high antilytic component and used amounts sufficient to neutralize the bacteriophage or lysins, thereby allowing regular and normal growth of the bacteria. He could not, under these conditions, detect a lysogenic action after repeated passage of susceptible bacteria. D'Herelle's experiments can be easily repeated. The only way to obtain his results is to have an excess of lysins. He used bouillon for contact during growth of the bacteria and subcultivated on agar slants. Bouillon cultures yield variable results, and the agar slant is not of uniform thickness and gives an untrustworthy test of lytic activity for quantitative work. We do not think he had inhibition as he interpreted his results, but he had an excess of lysins, and was able to demonstrate this excess when he had a sufficient concentration of these lysins to be detectable by the method he was using.

CERTAIN ENZYMATIC STUDIES OF BACTERIOPHAGE

Proteolysis.—Fresh moist fibrin, fresh egg white, coagulated egg white, coagulated egg white and coagulated blood serum were used as protein sources; 10 c.c. of undiluted strong bacteriophage, reaction adjusted to P_H 8.4 with phosphate buffered solutions, were put in each of the 16 tubes used in this experiment, 4 tubes for each of the proteins that served as substrate. Two tubes of each set were incubated for 48 hours at 37 C. under toluol; the remaining 2 tubes were heated in the water bath at 100 C. for 30 minutes to inactivate the bacteriophage, and served as controls. At the end of the incubation period, all tubes were heated at 100 C. for one hour to inactivate completely any enzymatic activity and evaporate the antiseptic used. After filtering, duplicate Van Slyke amino-nitrogen determinations were made of each sample. There was no increase in the amino acid nitrogen in the tubes containing the active bacteriophage and the proteins over that of the controls containing the inactivated bacteriophage. The lipolytic activity of the bacteriophage was tested in a similar set of experiments.

Olive oil and ethyl butyrate was used as substrate. The H-ion concentration of the controls and the incubated sets were the same.

From our experiments, we could not find that the bacteriophage had the power to hydrolyze certain proteins and fats. We have not been able to get sufficient quantities of the proteins and fats from the bacterium itself to determine whether there is a proteolytic or lipolytic activity manifested against these substances. Similar experiments have been carried out by many workers using toxins of various origins, and all of these have been, so far as we know, without evidence of enzymatic activity.

DISCUSSION

Northrop,¹⁰ in his studies of proteolytic enzymes (pepsin and trypsin), has shown that certain substances formed by their action on the substrate combine with the enzyme, and the equilibrium between the free and combined enzyme obeys the ordinary law of mass action. Since it is only the uncombined or dissociated ferment which causes further hydrolysis, the irregular results usually obtained under ordinary experimental conditions can be readily understood. If increased amounts of this intermediate split protein are added to the ferment solution, the first amounts added inactivate more ferment than later additions. This is a striking analogy to the manner in which antitoxin and toxin react in Ehrlich's experiments.

The standardization of the toxins are based on the uniform susceptibility of some animal of a given size; the antitoxin is standardized or evaluated in terms of the known toxin unit. The uncombined or active enzyme and the combined or inactive enzyme could be determined by the evidence of the hydrolytic activity on a known substrate; concentration, reaction, temperature and time are all controllable factors. The phenomenon of bacteriophagy only becomes demonstrable when the active lytic agent comes in contact with a growing susceptible bacteria.

It has been found that the active principle of the bacteriophage is most potent when added to a culture of susceptible bacteria during the period of logarithmic phase of growth of their life cycle. At this time there is a rapid increase in the potency of the active principle; later on there is a decrease in its activity.^{3, 11} When duplicate experiments are made in broth, the concentration of the lytic agent in the different tubes treated in the same manner and under the same conditions vary

¹⁰ Jour. Physiol., 1920, 2, p. 471; 1922, 4, pp. 227, 245 and 261.

¹¹ Doerr: Klin. Wchnschr., 1922, 1, p. 1489.

much. If 10 tubes containing exactly the same amount of broth are seeded with the same number of bacteria of as near as possible the same age and equal amounts of bacteriophage are added to each tube and incubated, the results are variable. After 6, 12, 24 or 48 hours some tubes are clear, some slightly cloudy and some as cloudy as the control bacterial growth. We have repeated such an experiment many times; one can never predict what the outcome will be. We are sure that any worker with experience in this field has observed the same thing many times. D'Herelle¹² mentions such variations, and admits there is probably an inhibiting factor present.

This variation in bacteriophagic concentration under as nearly as possible constant experimental conditions in fluid has been observed by all investigators. One can recall that enzymatic activity was equally puzzling before accurate chemical methods for the determination of the substrate and its split products were known.

Whether we have inhibition of the lytic principle or its actual destruction in fluid medium, is still an open question. The great variations in the lysogenic activity of mixtures of bacteriophage and susceptible bacteria in fluid medium, as compared with the more constant results on solid medium, has led us to suspect that there is possibly inhibitory substance produced under some conditions that inactivates the lytic principle in fluid medium. This is probably absorbed in the solid medium and does not play so important a rôle, thereby giving us more constant and comparable results. This point is now being investigated.

In all the bacteriophage work the complicating factor is the necessity of testing the potency or activity of our lytic agent against a growing susceptible micro-organism. Most all of the contradictory results recorded in the fast accumulating literature on this phenomenon are due to a lack of ability to evaluate and control the factors underlying growth of bacteria.

We have found that the lysin-antilysin reaction is comparable to that of the toxin-antitoxin in all of our experiments. Each batch of lysins must be carefully standardized, and also the antiserum must be titrated as to its antilytic content. The variations in the amounts of lysins-antilysin as shown in our tables are no greater than similar variations in toxins and their corresponding antitoxins obtained by other workers in this field.

¹² *Compt. rend. de soc. de biol.*, 1922, 86, p. 360.

SUMMARY

Susceptible bacteria grown in antilytic serum do not acquire immunity or resistance against the lytic activity of the bacteriophage.

Susceptible bacteria grown in antilytic serum do not decrease the antilytic power of this serum, but they cause a decrease in the agglutinins, opsonins and amboceptors for the homologous bacteria.

The union of lysin-antilysin in mixtures is varied by the same factors of time, temperature and Berkefeld filtration as the union of toxin-antitoxin in mixtures of similar concentration.

Lysin-antilysin union follows the laws of definite proportion as does toxin-antitoxin union.

Danysz-Bordet phenomenon of the toxin-antitoxin mixtures is manifested also by lysin-antilysin mixtures.

The antilysins belong to the same group of antibodies as antitoxins and anti-ferments.

THE ISOLATION OF SUBSTANCES WITH IMMUNE PROPERTIES

I. THE FRACTIONATION OF ISO-ELECTRIC AMBOCEPTOR PSEUDO- GLOBULIN BY ELECTRODIALYSIS

ARTHUR LOCKE* AND EDWIN F. HIRSCH

From the Pathological Laboratory of St. Luke's Hospital, Chicago

The substances with immune properties in antiserums as yet have not been isolated in pure form. The physical and chemical nature of these substances, as a consequence, is still a matter of conjecture, and the solution of the general problem of immunity depends, to some extent at least, on their isolation and chemical examination.

The early experiments of Pfeiffer and Proskauer¹ demonstrated that the serum of an animal injected with bacteria is able, after a time, to agglutinate other bacteria of the same strain. Sewall,² in this country, found that the serum of rabbits which have been injected with small quantities of snake venom contains a substance capable of neutralizing the venom poison. These are properties not manifested to the same degree by the serum of normal animals. A large number of similar and corroborative observations has followed, and many attempts have been made by different methods to isolate in pure form, the substances possessing the specific immune properties.

The immune reactions, as for example the agglutination of bacteria, belong to the general class of heterogeneous or colloidal reactions, and blood serum, the raw material from which the immune substances are recovered, is essentially a colloidal solution. The isolation of immune substances, therefore, becomes largely a problem in colloidal chemistry. The successful isolation of these substances in pure form now seems likely because of electrodialytic methods recently devised³ for fractionating serum globulins.

The immune substances are neither dialyzable nor are they extractable from serum by means of the usual fat solvents. They apparently are associated with the nonfatty or protein colloids of the serum. On

Received for publication, Sept. 3, 1924.

* The Seymour Conan Fellow in Chemistry, Kent Chemical Laboratory, University of Chicago.

¹ *Centralbl. f. Bakteriol.*, 1896, 19, p. 191.

² *Jour. Physiol.*, 1887, 8, p. 203.

³ Locke, A. P.: *Jour. Biol. Chem.*, in print.

the basis of Kauder's⁴ fractionation of serum proteins by half-saturation with ammonium sulphate—that part precipitated being known as globulin, that remaining in solution as albumin—Pfeiffer and Proskauer¹ found all of the agglutinating properties of cholera immune serum contained in the globulin precipitate. Corroborative observations quickly followed, verifying the association of the immune substances with the globulin fractions in diphtheria immune serum,⁵ in tetanus antitoxin,⁶ in hemolytic serum for sheep corpuscles,⁷ and recently others have established the globulin nature of the substance in the serum of syphilitic patients responsible for the positive reaction with the Wassermann test.⁸

When division of the globulins into euglobulins and pseudoglobulins was made by Fuld and Spiro⁹ on the basis of insolubility or solubility in a saturated solution of sodium chloride, certain of the substances with immune properties were demonstrated in one or the other of these fractions.¹⁰ Pick reports the diphtheria antitoxin in the pseudoglobulin fraction of immune goat serum; the typhoid agglutinin in the euglobulin fraction of immune goat, rabbit, and guinea-pig serums, but in the pseudoglobulin portion of immune horse serum. Curiously, he finds the cholera agglutinin in the euglobulin fraction of immune horse and goat serums. Rodhain¹⁰ reports the agglutinins for streptococci in the euglobulin fraction of immune rabbit serum. Fuhrman,¹⁰ and subsequently Kurt Meyer,¹⁰ found the hemolysins contained in the pseudoglobulin fraction. The pneumococcus antisubstances and the opsonins are distributed among the pseudoglobulin and euglobulin fractions. From these observations and from comments by the authors mentioned, the conclusion may be reached that the substances in antiserums with immune properties are contained in the pseudoglobulin fraction or in the closely bordering euglobulin fractions of the immune serum.

Antiserums for the treatment of diphtheria, tetanus, meningitis, pneumonia, and streptococcus infections, are prepared commercially on a large scale in horses. Care in injecting these serums into patients

⁴ Arch. f. exper. Path. u. Pharmacol., 1886, 20, p. 415.

⁵ Pick: Beit. z. chem. Phys. u. Path., 1902, 1, p. 351.

⁶ Tizzoni and Gattani: Zentralbl. f. Bakteriol., 1891, 9, p. 10. Emmerich and Tsuboi: Die Natur der Schütz u. Heilschubstanz des Blutes, 1892.

⁷ Landsteiner: Zentralbl. f. Bakteriol., 1900, 27, p. 357.

⁸ Kapsenberg: Ann. d'Inst. Pasteur., 1921, 35, p. 648.

⁹ Zeitschr. f. physiol. Chem., 1900, 31, p. 140.

¹⁰ Seng, Zeitschr. f. Hyg. u. Infektionskrankh., 1899, 31, p. 513. Fuhrman: Beitr. z. chem. Physiol. u. Path., 1903, 3, p. 417. Meyer: Arch. f. Hyg., 1908, 67, p. 114. Rodhain: Beitr. z. chem. Physiol. u. Path., 1902-03, p. 451. Avery: Jour. Exper. Med., 1015, 21, p. 133. Zinsser: Jour. Immunol., 1921, 6, p. 889.

is necessary because of serum disease, brought about through sensitization by a preceding injection of horse serum, not infrequently given for treatment of, or immunization against, a disease different from the one being combated with the antiserum. The minute quantities of horse serum used in the immunization with diphtheria toxin-antitoxin mixtures have been observed by Hooker¹¹ and Park¹² to sensitize human adults and children for considerable periods of time. This danger exists because of the protein content of the whole serum. Fractional precipitation methods have been devised with the object of removing as much of the inert protein substances as possible, and of retaining and of concentrating the immune fractions.

Ammonium sulphate precipitation is used largely in the commercial concentration and refining of immune serums.¹³ Gibson, Banzhaf, and Heinemann have established excellent methods for the purification of antitoxin serums, which depend on a fractional precipitation with ammonium sulphate of globulins from albumin, and on further separation of the pseudoglobulin from the euglobulin with a saturated solution of sodium chloride. A partial denaturation of the resulting pseudoglobulin solutions by preliminary heating to 50-70 C. reduces their antigenic effect. Serum disease with the use of these preparations is reported to be considerably less frequent than with injections of the whole immune horse serum.

Methods for the purification of immune serum by evaporation and freezing have not been entirely successful.¹⁴ Fractionation of proteins in normal serum by ultrafiltration was attempted by Mannich.¹⁵ This method of fractionation was attempted also by the authors with immune serums, but without success.

With the development of methods for the purification of immune serum based on precipitation with ammonium sulphate, there have grown to commercial importance and significance methods based on dialysis. Panum,¹⁶ in 1852, reported that the colloidal serum proteins flocculate when the small amounts of electrolyte present and necessary for their dispersion are depleted by dilution or dialysis. The fraction precipitating

¹¹ Jour Immunol., 1924, 9, p. 7.

¹² Ibid., p. 17.

¹³ Gibson: Jour. Biol. Chem., 1906, 1, p. 168; 1907, 3, p. 233. Banzhaf: Coll. Studies, Research Lab., Dept. of Health, New York City, 1908-09, 4, p. 230; 1912-13, 7, p. 114. Heinemann: Jour. Infect. Dis., 1916, 19, p. 433. Brieger and Krause: Berl. klin. Wehnschr., 1907, 10, p. 30. Besredka: Ann. de l'Inst. Pasteur, 1923, 37, p. 935. Zeissler: United States Patents, Number 1,472,316, Oct. 30, 1923. Frouin: Compt. rend. Soc. de biol., 1908, 65, p. 444.

¹⁴ Bujwid: Zentralbl. f. Bakteriol., 1892, 1, p. 12. Ernst, Coolidge and Cook: Jour. Boston Med. Soc., 1898, 2, p. 166. Hata: Zentralbl. f. Bakteriol., 1909, 1, p. 48.

¹⁵ Ztschr. f. Nahrungsm., 1920, 40, p. 12.

¹⁶ Virchows Arch. f. path. Anat., 1852, 4, p. 32.

was shown by Aronstein¹⁷ and Schmidt¹⁸ to be euglobulin. The application of this method to the purification of immune serums has not been extensive because of the many practical difficulties and disadvantages with its use.¹⁹ The addition of an electrolytic process to slow dialytic diffusion²⁰ causes a much more rapid flocculation of the protein, a matter of importance where stability of these substances is a factor, and further makes possible the flocculation of pseudoglobulin,²¹ which cannot be accomplished to any appreciable extent by simple dialysis. The apparatus which was developed at first for the electro-dialysis of protein solutions,²² and which was modified later for the purification of immune serums by the Elektro-Osmose Gesellschaft (Schwerin), consists²³ of a horizontal tube divided into 3 equal compartments by 2 semipermeable membranes. The serum is contained in the central compartment, and the end compartments are filled with distilled water. They also contain the platinum electrodes. When a voltage drop has been established between these electrodes, the ions of the serum electrolyte are attracted into the cathode and anode chambers, the equilibrium between the crystalloids and the proteins within the serum is shifted outside of the narrow zone of stable dispersion and globulin flocculates. It has been emphasized that the entire success of the method depends on maintenance in the serum compartment of a reaction approximately P_H 7. This seems to have been obtained only by the choice of suitable membranes, cellulose parchment for the cathode and a protein parchment for the anode. Should the reaction of the serum solution become markedly acid or alkaline, the protein substances are denatured, and there is also a loss of the immune activity.

While Pauli reports that he has accomplished the precipitation of normal serum pseudoglobulin with long-continued electro-dialysis, it is not clear from any published work that unmodified "immune" pseudoglobulin has been precipitated in this way. Purified solutions of pseudoglobulin have been prepared, so far, by flocculation and removal of the more easily precipitated euglobulin. Flocculation of the pseudo-

¹⁷ Pflüger's Arch. f. Physiol., 1874, 8, p. 75.

¹⁸ Beitr. z. Anat. u. Physiol. als Festgabe Ludwig gewidmet, 1874. Heynsius: Pflüger's Arch. f. Physiol., 1876, 12, p. 549.

¹⁹ Felton: Science, 1924, 59, p. 433. De Kruif and Eggerth: Jour. Infect. Dis., 1919, 24, p. 505.

²⁰ Dhéré: Compt. rend. Soc. de biol., 1910, 150, p. 934; Jour. phys. path. gen., 1912, 13, p. 157.

²¹ Pauli: Klin. Wchnschr., 1924, 3, p. 1.

²² Pauli (Footnote 21). Adolph: Kolloid Chem., Beihefte, 1923, 17, p. 1. Stern: Centralbl. f. Bakteriol., 1923, 75, p. 573.

²³ British Patents, Number 104,688, 1917; Number 146,260, 1920. Ruppel: Berl. Pharm. Ges., 1920, 30, p. 314; Ztschr. f. Hyg. u. Infektionskrankh., 1922, 97, p. 188.

globulins by electro-dialysis, however, seems not to have been accomplished to any extent. The reason for this, perhaps, is the emphasis placed on maintaining the reaction of the dialyzing serum at P_H 7. We have found essential that this reaction be held near P_H 5, a reaction slightly more alkaline than the iso-electric point, or P_H of minimum solubility, of the pseudoglobulin. The regulation of the P_H through

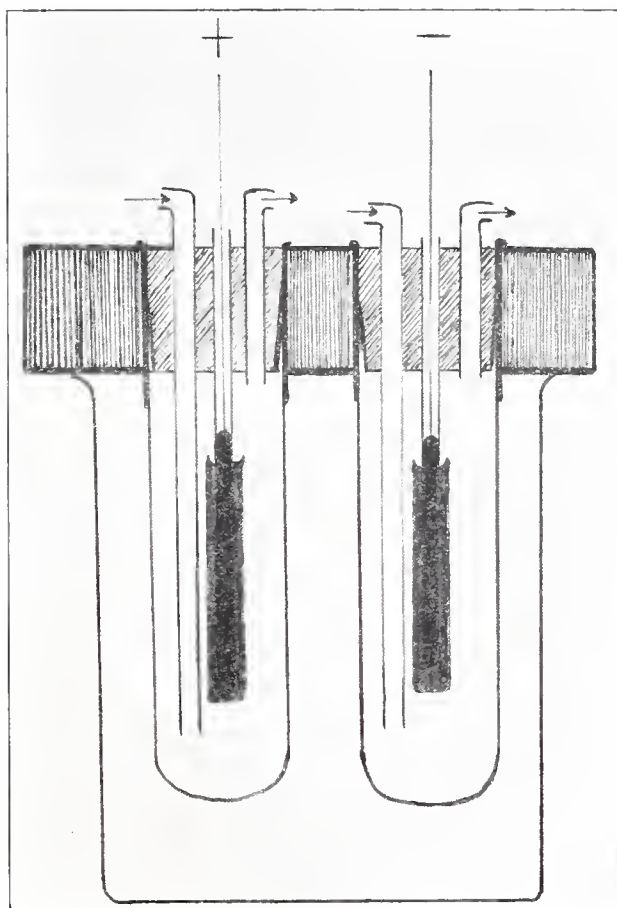


Fig. 1.—Apparatus for electro-osmosis.

the choice of membranes is exceedingly difficult. This difficulty may be avoided, and the desired regulation of the P_H obtained by using the apparatus and the procedure described below.

The electro-dialyzing apparatus is prepared by softening in water two 16 x 100 mm. (Schleicher and Schull) dialyzing thimbles. The open end of each is fitted with a 3 hole, rubber stopper through which pass 2 tubes for the water circulation, and a mercury filled tube into which is sealed a platinum foil electrode, 1 x 4 cm. (Fig. 1). Each thimble is pushed into a flanged glass

collar 4 cm. long, so that the rubber stopper, when in place, makes a water tight connection. The 2 dialyzing thimbles, so prepared, are mounted in a large cork stopper. The platinum electrode foil is bent back over the mercury filled tube and is wrapped with parchment to prevent corrosion of the dialyzing thimble during electrolysis. The proper water and electric connections are completed. The dialyzing thimbles are then immersed in 55 c.c. of the serum solution contained in a 100 c.c. beaker. Electrolysis is commenced only after there is established a vigorous circulation of cold tapwater, and at a potential drop of 25 volts. At no time should there be heating of the serum by internal electrical resistance. Experiments have demonstrated that the use of circulating tapwater (rather than distilled water) at the beginning of the electro-dialysis maintains the reaction of the serum at a safe alkalinity. This is necessary because an excessive acidity or alkalinity alters the proteins and causes also a loss of immune activity. The accumulation of insoluble salts in the serum solution during this procedure is not objectionable.

For the electrodialytic fractionation of the globulins, advantage is taken of the greater conduction of the electrical current along the surface of the solution. Removal of electrolyte takes place more rapidly here as a consequence, and the least stable of the peptized globulin particles flocculate. This precipitate settles and redissolves in the electrolyte—richer and more alkaline layer below, thereby increasing the globulin content of this portion. As the clear zone proceeds downward, its content in almost pure pseudoglobulin increases. The clear liquid zone is removed with a clean pipet, and is transferred into another similar electro-dialyzing unit through which ice chilled, distilled water circulates. Electrolysis is continued at 50-80 volts. The reaction of the solution becomes approximately P_H 5, and as dialysis progresses, iso-electric pseudoglobulin settles at the bottom of the beaker, forming a markedly viscid, gelatinous, transparent, light yellow mass. This crude precipitate is redissolved and reflocculated by electro-osmosis against distilled water and at 110 volts.

Antisheep amboceptor of high titer, prepared in rabbits, was used in the experiments mentioned in this report. This system was chosen because of the accuracy and speed with which immune substance content can be determined in unit values. Such evaluations are important with serum fractionation, because the immune content of each portion separating must be tested in order to determine where the substance with immune properties is contained. The results of such tests point out the fractions to be discarded, and those to be retained. The system also enables the detection of any loss in potency occurring with manipulation. The unit of amboceptor used in these experiments is the amount required to hemolyze completely 0.5 c.c. of a 5% sheep cell suspension in the presence of one unit of complement (determined by previous titration) within 30 minutes' incubation, the total volume being made 2 c.c. with 0.9% salt solution.

By the electrodialytic method described, the entire potency of the original immune serum has been conserved and recovered almost quantitatively in the final yield. Amboceptor serum containing originally about 2,000 units per c.c. yields 150,000 to 200,000 units per gram of the freshly dried, water-soluble pseudoglobulin. The activity of the substance obtained depends on the original potency of the serum before concentration, and the extent of the fractionation to which it has been

subjected. No claims are made that even these preparations represent the purest obtainable fractions. They do exceed in strength, however, those prepared by Hirsch and LeCount.²⁴

The dried immune pseudoglobulin purified by electrodialysis gradually loses its solubility in water on long storage in sealed ampules, and there is a parallel loss in its potency. This loss in potency is assumed to follow a change in the physical nature of the protein, a denaturation because of a gradual loss of water content. This is somewhat inhibited when the protein is preserved as an alkaline salt. There is hope of its being entirely prevented by converting the protein into another compound.

SUMMARY

The isolation from serum of immune iso-electric pseudoglobulin (amboceptor) of high titer has been accomplished by means of an electrodialytic procedure, which is described.

The amboceptor can be isolated quantitatively by this method, and the titer of freshly dried amboceptor preparations is 150,000 to 200,000 units per gram.

Sealed in ampules, the dried iso-electric pseudoglobulin is not stable indefinitely, but loses its solubility in water and coincidentally its potency.

Although these preparations are more pure than any so far prepared, they are not regarded as representing the pure amboceptor substance.

²⁴ Jour. Infect. Dis., 1924, 34, p. 103.

SURFACE TENSION OF CULTURE MEDIUMS

M. S. MARSHALL

From the Michigan Department of Health, Lansing

The purpose of this paper is to present experimental data relative to the surface tension of mediums, with some experiments on bacterial growth in mediums of different surface tension. We have confidence in the accuracy of the data; it has seemed, however, that the conclusions to be drawn are more by way of suggestion than by way of proof of particular points. The paper will, for this reason, be limited to the presentation of the data to the exclusion of statements regarding the possible bearing on related questions.

EXPERIMENTAL

The du Noüy Tensiometer.—This instrument¹ for the measurement of surface tension was devised in 1919. It is a simple torsion balance on which is hung a platinum-iridium ring, the pull of which in separating from a liquid is measured as a function of the torsion applied. The instrument is now on the market with a dial reading directly in dynes per cm.; our instrument (Central Scientific Company, No. 250) is graduated by an arbitrary scale and required calibration. After experimenting with the weight method of calibration, determining the torsion pull by weights, with pure substances, it was found that, although the weighing process is, of course, accurate, such calibration did not seem to agree with all instruments. In the Smithsonian Tables, water is given a surface tension of 72.8 dynes per cm. at 20 C. and 72.1 at 25 C. Du Noüy's figure is 77 dynes per cm. at 25 C., and for our instrument 75.2 at 23 C., using the weight method. We hence used redistilled water in a large number of trials, and at intervals to check any possible influence of strain on the torsion wire, with the result that 0.691 ± 0.004 dial unit was found to represent 1 dyne per cm., based on the Smithsonian Tables figures for water.

The Traube Stalagmometer.—This generally known form of surface tension apparatus depends on the size of a drop falling from a definite surface at a slow rate. We made such a standard instrument, blowing the bulb in a 0.2 c.c. pipet graduated at 0.001 c.c. intervals, and polishing the end of heavy capillary tubing for the dropping surface. By mounting at the upper end of the apparatus a stop-cock and a glass tube sealed with the exception of a microscopic hole, the rate of dropping was easily controlled, and fractions of a drop were easily measured by the calibration above and below the bulb.

Calibration of the instrument gave 72.15 ± 0.10 drops of water per unit volume between the marks on the instrument or

$$\alpha_x = 5234 \cdot \frac{\Delta_x}{n_x}$$

where α_x indicates the surface tension of the unknown liquid, Δ_x its density, and n_x is the number of drops for the calibrated volume.

¹ Du Noüy: Jour. Gen. Physiol., 1919, 1, p. 521.

Comparison Between Ring and Drop Methods.—The surface tension of beef infusion broth medium, beef extract broth medium, and salt-free beef infusion broth was measured with the tensiometer (static) and with the stalagmometer (dynamic) at 23 C. (table 1).

TABLE 1
SURFACE TENSION BY STATIC AND DYNAMIC METHODS

	Static	Dynamic	Difference
Beef infusion broth.....	46.2*	54.4	+8.2
Beef extract broth.....	52.3	58.9	+6.6
Salt-free infusion broth.....	50.0	56.4	+6.4

* Figures are given throughout in dynes per cm.

The densities were respectively, 1.011 at 23.75 C., 1.006 at 24.0 C., and 1.008 at 23.75 C.

A castor oil soap solution, made according to Larson,² used in subsequent experiments to reduce the surface tension as required, was measured for surface tension in different concentrations in aqueous solutions with distilled water (table 2).

TABLE 2
MEASUREMENT OF SURFACE TENSION OF AQUEOUS CASTOR OIL SOAP SOLUTIONS

% Soap Solution	Static	Dynamic	Temperature, C.
0.00	72.7	72.7	21.0
0.02	48.2	66.8	21.0
0.10	40.1	56.9	21.0
0.20	38.0	48.6	21.0
0.50	35.8	38.9	21.4
1.00	34.9	36.2	21.0

The same procedure was adapted to the variation of surface tension with soap concentration in beef infusion broth (table 3).

TABLE 3
MEASUREMENT OF SURFACE TENSION OF BEEF INFUSION BROTH SOAP SOLUTIONS

% Soap Solution	Static	Dynamic	Temperature, C.
0.00	45.6	60.2	20.0
0.05	35.2	42.15	20.2
0.10	34.5	38.25	20.6
0.20	34.2	35.3	21.4
0.50	34.2	34.0	21.8
1.00	34.3	32.0	21.8

² Jour. Infect. Dis., 1919, 25, p. 41.

Variation of Surface Tension with Concentration of Medium Ingredients.—The relationship between surface tension and concentration of peptone (Difco) in distilled water was followed, using the tensiometer (table 4).

TABLE 4
RELATION BETWEEN SURFACE TENSION AND CONCENTRATION OF PEPTONE IN DISTILLED WATER

% Peptone	Surface Tension	Temperature, C.	Exper. No.
0.000	72.5	23.0	1
0.000	72.5	23.0	2
0.033	57.9	22.0	4
0.076	67.2	23.0	2
0.091	64.4	23.0	2
0.100	63.0	21.7	5
0.167	62.8	23.0	2
0.192	60.8	22.5	3
0.192	59.2	22.5	3
0.192	59.5	22.5	3
0.192	62.8	22.0	4
0.250	66.1	23.0	1
0.333	58.8	23.0	2
0.370	62.8	22.5	3
0.370	60.9	22.0	4
0.455	60.4	22.5	3
0.455	59.9	22.0	4
0.500	57.1	23.0	1
0.500	58.2	23.0	2
0.750	56.8	23.0	1
0.833	58.0	22.5	3
0.833	61.8	22.5	3
0.833	58.0	22.5	3
1.000	56.8	23.0	1
1.000	56.7	23.0	2
1.667	57.0	22.5	3
1.667	54.5	22.0	4
2.000	56.0	23.0	1
5.000	53.0	23.0	1
5.000	53.2	22.5	3
5.000	53.1	22.0	4

Similar variation in the surface tension of aqueous solutions of beef extract (Difco) was studied (table 5).

TABLE 5
SURFACE TENSION OF AQUEOUS SOLUTIONS OF BEEF EXTRACT

% Beef Extract	Surface Tension	Temperature, C.
0.00	72.2	24
0.01	63.1	24
0.05	57.6	24
0.10	54.6	24
0.50	51.1	24
1.00	49.1	24
5.00	43.6	24
10.00	42.4	24

The variation in surface tension with concentration of NaCl in aqueous solution is virtually nil (table 6).

TABLE 6
SURFACE TENSION OF NaCl IN AQUEOUS SOLUTION

% NaCl	Surface Tension	Temperature, C.
0.00	72.3	23.2
0.02	67.8	23.0
0.10	68.6	23.0
0.40	67.8	22.6
1.00	70.5	21.8
10.00	70.5	21.8

From 15 to 30 minutes were necessary in reaching equilibrium conditions for constant readings with the tensiometer. The table represents the results of 94 determinations.

The surface tension of an ascitic fluid which had been filtered through a Berkefeld filter was measured in various concentrations in water (table 7).

TABLE 7
SURFACE TENSION OF ASCITIC FLUID IN AQUEOUS SOLUTION

% Ascitic Fluid	Surface Tension	Temperature, C.
0.00	72.5	23.0
0.05	69.1	22.5
0.10	63.0	22.9
0.50	64.0	23.0
1.00	59.7	23.1
5.00	58.8	23.3
10.00	58.8	23.2

TABLE 8
SURFACE TENSION OF BEEF INFUSION BROTH CONTAINING SOAP SOLUTION IN DIFFERENT CONCENTRATIONS AND SIMILAR BROTH TO WHICH 2% OF ASCITIC FLUID HAD BEEN ADDED

% Soap	Plain Broth	Ascitic Broth	Temperature, C.
0.000	45.6	45.8	23
0.055	35.0	35.1	23
0.111	34.8	34.6	23
0.170	34.2	34.2	23
0.222	34.2	33.9	23

Dextrose was similar in its effect on broth surface tension.

Using systematic aqueous dilutions of beef infusion broth, the surface tension varied in the following manner:

TABLE 9
VARIATION IN SURFACE TENSION WITH SYSTEMATIC DILUTIONS OF BEEF INFUSION BROTH

% Broth	Surface Tension	Temperature, C.
0.0	72.7	23
0.1	63.2	23
1.0	52.3	23
5.0	50.5	23
10.0	49.7	23
50.0	44.9	23
100.0	44.2	23

The Surface Tension of Various Mediums.—The tensiometric surface tension of various liquid mediums was measured. The plus or minus quantities represent variation from the mean found when a number of different lots of mediums were tested.

TABLE 10
SURFACE TENSION OF VARIOUS MEDIUMS

Medium	Surface Tension	Temperature, C.
Beef infusion broth.....	45.4 ± 1.9	23.0
Beef extract broth.....	51.9 ± 0.9	23.0
Salt-free infusion broth.....	47.6 ± 2.4	23.2
Dextrose infusion broth.....	45.1 ± 0.5	22.0
Toxin veal infusion broth.....	45.6 ± 2.0	24.0
Dunham's peptone solution.....	52.8	22.8
Loeffler's uncoagulated serum.....	54.1 ± 2.2	23.0
Milk, litmus.....	47.0	23.0
Synthetic medium ^c	70.0*	23.0
Agar †.....	44.2	22.0
Gelatin †.....	44.7	22.0
Whole bile.....	46.4	22.3
Difco 10% bile (fresh).....	42.2	22.4
Difco 10% bile (3 months).....	42.2	22.3
Broth + :.....	45.2	21.4
CH ₃ COOH.....	40.6	21.5
HCl.....	41.6	21.7
CuSO ₄	42.7	22.0
KCl.....	45.0	22.0
Glycerine.....	45.6	22.0
Brom thymol blue.....	43.1	22.2
NaOH.....	46.8+	22.0
CaCl ₂	46.2	22.3
CaCl ₂ + NaOH.....	46.9	22.3

* Filtered sugars do not change appreciably.
† Weak concentrations, not solidified.

Rate of Development of B. Dysenteriae Shiga in Beef Infusion Broth, P_H 7.2–7.4, at 37 C.—If a young rejuvenated culture be transferred during the logarithmic phase of growth to a similar medium at 37 C. it follows that the log of the bacterial concentration plotted against time is a straight line, and is a function of the time *t* elapsing between binary fission.* The slope is (log 2) /*t*, and hence a change in rate of

$$C = N 2^{\frac{T}{t}}$$
$$\text{or } \log C = \log N + \frac{T}{t} \log 2$$

* Bacteria per c.c. is indicated by C; organisms per c.c. at start N; time from inoculation in hours T, and the time of fission, t.

division will be manifested by a difference in slope. The normal rate for *B. dysenteriae* Shiga is given in fig. 1, in which t is 31.3 minutes, the variation being in the experiments charted from 29.4 to 34.0 minutes.

Experiments were made on the normal rate of *B. dysenteriae* Shiga in ordinary infusion broth, as compared with the rate in a 0.2% soap solution broth having a surface tension of 33.9 dynes per cm. (normal was 45.8 ± 0.5). Although there was a definite variation from the normal, it fluctuated considerably, seeming to follow no semblance of a law. There must be some consistent variation which we did not find. The fluctuations noted were so marked that the data is considered useless in this connection, except for the general statement that a lowered surface tension tends to lower the rate.

Variation in Gas Metabolism of B. DYSENTERIAE Shiga with Surface Tension.—For this work, the manometer devised by Dr. F. G. Novy,³ slightly modified to provide a rest for his "h-tube," as shown in fig. 2 was used for the measurement of the pressure. The gas was removed from the h-tube while still attached to the manometer, and analyzed for CO₂ and O₂ in the Haldane-Henderson⁴ apparatus, small model for 7 to 10 c c. of gas. The CO₂ and O₂ content of the medium was not measured.

TABLE 11
VARIATION IN GAS METABOLISM

% Soap Solution	Surface Tension, Temperature 23 C.	Age When Analyzed	%CO ₂	%O ₂
0.000	45.0	25.0 hours	3.88	16.25
0.000	45.0	29.0 hours	2.33	17.92
0.000	45.0	216.0 hours	12.91	4.34
0.000	45.0	Not inoculated	0.03	20.61
0.000	45.0	Not inoculated	0.02	21.07
0.049	35.5	24.0 hours	1.93	18.81
0.098	34.5	27.5 hours	2.36	18.45
0.194	34.5	23.0 hours	1.34	20.01

The manometric pressure is negligible over a 24-hour period, but reaches — 21 mm. in a week, and — 25 mm. in 9 days.

Change in Surface Tension During Growth.—There is no change in surface tension during the growth of various colon-typhoid organisms tested in plain or dextrose beef infusion broth, or in plain or dextrose

³ In press.

⁴ Henderson: Jour. Biol. Chem., 1918, 33, p. 31.

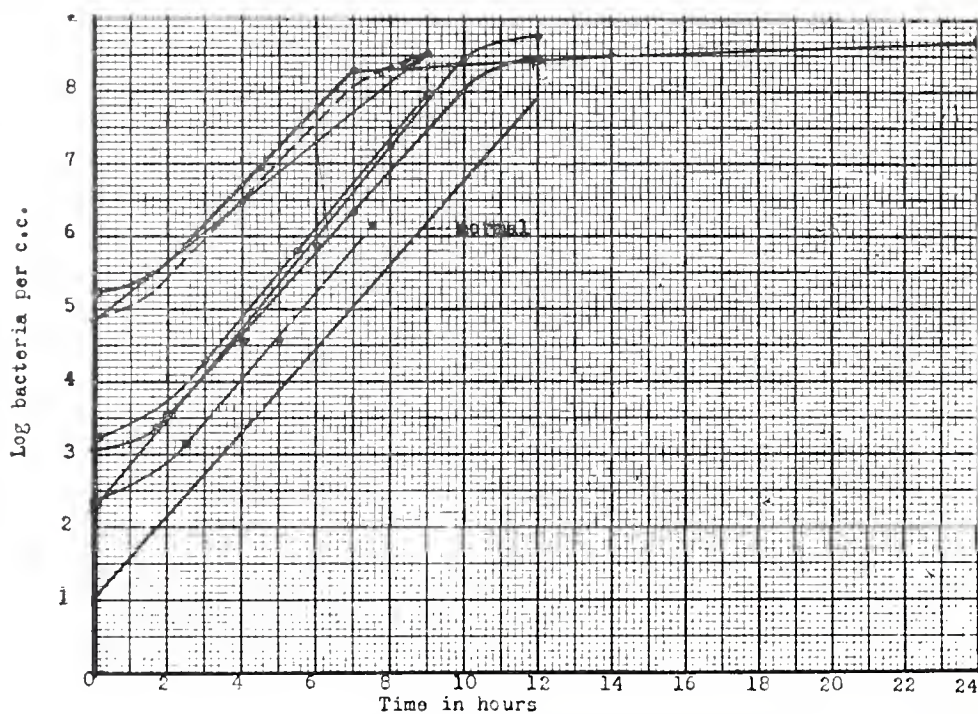


Fig. 1.—Rate of multiplication of *B. dysenteriae* Shiga.

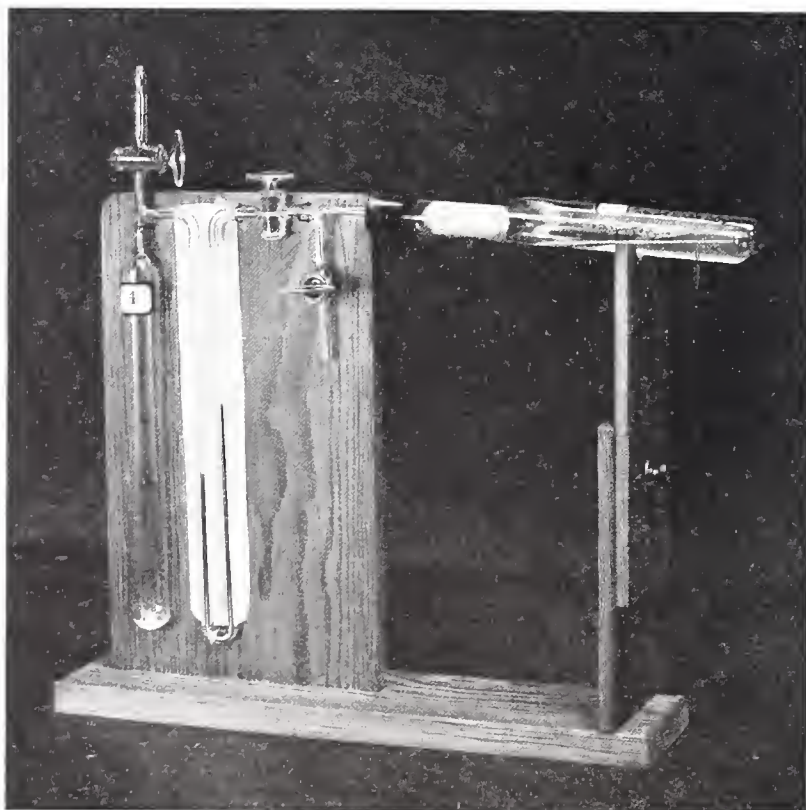


Fig. 2.—Manometer devised by Dr. F. G. Novy.

synthetic medium.⁵ A concentration of 20,000,000 *B. coli* organisms per c.c. means approximately only a 0.004% suspension or solution of cellular material, and the additive effect of this concentration of cellular material and metabolic changes brought about in the medium does not change the surface tension of the medium over a period of 36 hours.

The effect of inoculation from broth culture to a medium on its surface tension is hence obviously negligible when broth is used, and with the synthetic medium of nearly the surface tension of water, the surface tension is changed in proportion as the amount of broth added with inoculation, in accordance with the data given for broth concentration in water as a function of the surface tension. It was, however, possible to inoculate the synthetic medium from an agar slant in considerable amounts without altering the surface tension in any way.

Synthetic Mediums of Different Surface Tension.—It is possible by means of the castor oil soap solution to make the synthetic medium so as to have a surface tension of from 35 to 70 dynes per cm.

TABLE 12
VARIATION IN SURFACE TENSION OF SYNTHETIC MEDIUM WITH SOAP CONCENTRATION
Temperature, 23 C. \pm 1

% Soap Solution	Surface Tension	% Soap Solution	Surface Tension
0.00	69.8	0.010	48.2
	70.0		48.2
	70.1		48.9
	71.2		49.1
	71.2	0.015	47.0
	71.2	0.050	39.4
	71.3		38.8
	71.4	0.100	35.9
	71.6		35.9
	71.8		35.9
0.001	58.4	0.200	35.2
0.005	50.3		35.2
0.010	47.8		34.9
	48.0		

These data are compiled from various experiments, and show the accuracy of duplication with different lots of synthetic medium, some without sugar and some with 1% dextrose, made up with soap solution at different times.

⁵ Ayers, Rupp and Johnson: U. S. Dept. of Agric. Bull. 782.

TABLE 13
RESULT OF PLATING B. COLI GROWN IN SUCH MEDIA

	% Soap	0.00	0.01	0.05	0.10	0.20
I	24 hours	1.186×10^6	9.78×10^6	15.01×10^6	20.4×10^6	11.12×10^6
II	0	345,800	345,800			
	3	9,450	7,875			
	6	1,890	500			
	22	<200	<100			
III	0	4,914,000	5,027,000			
	4	2,355,000	2,345,000			
	6.5	1,455,000	555,000			
	25	<10,000	<10,000			

In this study, the decrease in II and III would indicate that the synthetic base could scarcely be called a medium. Later study showed a very heavy inoculation to be necessary.

Using this knowledge, the pressure was measured over carbohydrate-free synthetic medium (table 14).

TABLE 14
RESULT WITH CARBOHYDRATE-FREE SYNTHETIC MEDIUM

	B. coli	K-84 *	B. subtilis	B. typhosus, Eberth
1 day	+12 mm.	+20	+11	6
4 days	+12 mm.	+36	+11	6

* One of Dr. R. R. Mellon's variants.

The B. typhosus culture, although checked to grow weakly in this medium, obviously failed here.

TABLE 15
RESULT WHEN USING A 1% DEXTROSE SYNTHETIC MEDIUM AND B. COLI AND REDUCED SURFACE TENSIONS

% Soap	0.0	0.0 Control	0.001	0.005	0.010	0.015	0.015 Control	0.050
1 day	+5.8 mm. +18.2 +15.3	+9.7	+10.5	+16.7	+17.8 +20.2 +13.3	+23.2	+13.7	+13.8
2 days	+5.8 +17.7		+11.2		+17.8 +14.2			+13.8
3 days	+18.0		+12.5		+18.0			

Analyses

% Soap	0.0		Control		0.001		0.005		0.010		0.015		Control	
	CO ₂	O ₂	CO ₂	O ₂	CO ₂	O ₂	CO ₂	O ₂	CO ₂	O ₂	CO ₂	O ₂	CO ₂	O ₂
25.5 hrs.	3.48	17.48
27.5	3.60	17.94
28.5	3.10	18.22
29.5	2.77	18.47
1 day	0.12	26.90	0.20	20.88
3 days	3.24	17.91	3.56	17.49	3.58	17.11

This particular synthetic medium has not given in our hands the consistency and stability to be desired for this particular work, although it does allow a range of surface tension between 35 and 70 dynes per cm., whereas beef extract broth raised somewhat by the addition of serum is the medium of high nutrient qualities having the highest surface tension of those measured, 54.1 dynes per cm.

DISCUSSION

The experimental data which have been given afford a fair basis for an understanding of the surface tension of broth mediums, and the rôle played by the elements entering into such mediums as affecting the surface tension.

Comparison with the work of Larson² will reveal that the drop method used by him gives results similar to our stalagmometric data, although not in accordance with the tensiometric data. Comparison between the two sets of data shows clearly that surface tension is differently manifested in the two methods, as is well known, so that the discrepancies are not real. This point has been adequately dealt with by du Noüy,⁶ in a paper surveying the field. General pertinent works might perhaps be given as Willows and Hatschek,⁷ Ferguson,⁸ and Harkins,⁹ although there are numerous valuable papers on the subject. Suffice it to say that since the surface tension of such colloidal material as we are dealing with is to a greater or lesser extent a function of time, the tensiometer method was adopted after the preliminary stalagmometer comparisons. And although it is true that surface tension has elements of being a physical constant, the method of measurement is all-important in comparisons of results.

Larson has made certain qualitative points in his surface tension work as regards pellicle formation and bacterial growth which are very suggestive. Lobes¹⁰ has given certain points on the gas formation of *B. coli* in protein-free medium. Hotchkiss¹¹ has determined the effect of certain cations on bacterial growth. Ayer, Rupp, and Johnson¹² have used mediums of varying surface tension with streptococci. A recent article by Green and Halvorson¹³ is of interest in connection with the problems.

⁶ J. Exper. Med., 1922, 35, p. 575.

⁷ Surface Tension and Surface Energy, 1923.

⁸ Sc. Prog., 1914-15, 9, p. 428.

⁹ Jour. Am. Chem. Soc., 1916, 38, p. 228.

¹⁰ Bioch. Zeitschr., 1922, 130, p. 1.

¹¹ J. Bacteriol., 1923, 8, p. 141.

¹² J. Infect. Dis., 1923, 33, p. 22.

¹³ Ibid., 1924, 35, p. 5.

The approach made in this paper has been toward quantitative systematic relationships. As regards culture mediums and surface tension, some data may be of constructive value. As regards the relationship between surface tension and bacterial growth, the data can be considered as little more than suggestive. After all, the relationship between the surface tension at the air medium interface is not necessarily a function of the surface energy relationships at the organism-medium interface in any way, and it may be that the more or less negative evidence presented herein points toward such a lack of correlation.

SUMMARY

A systematic study of the surface tension of liquid culture mediums has been made. Nine standard mediums have a surface tension within a range of 44 to 54 dynes per cm., some 20 dynes per cm. less than the surface tension of water. Bile, and agar and gelatin in nonsolidifying concentration fall within the same range. The effect of various inorganic and organic substances, exclusive of soap solutions, oils, and the like, is not great on the surface tension of infusion broth.

There occurs a marked reduction of the surface tension of distilled water by very small amounts of peptone, beef extract, ascitic fluid, castor oil soap solutions, and infusion broth; a minimum is reached at a low concentration of these substances. This is not true of dextrose solutions nor of NaCl solutions.

Comparison of static and dynamic measurements of surface tension gives radically different figures for the surface tension.

There results no change in surface tension of infusion broth nor of a synthetic medium as a consequence of bacterial growth.

Organisms growing in mediums of different surface tensions appear to have different rates of growth, and different gas metabolisms. Correlation of these variables, however, reveals no systematic variation.

SPECIFIC "RESIDUE ANTIGENS" OF DIFFERENT TYPES OF MENINGOCOCCI

FELIX PRZESMYCKI

From the Hygienic Laboratory, U. S. Public Health Service, Washington, D. C.

Zinsser and Parker¹ claim that in recent investigations they were able to obtain from different bacteria substances that give specific complement-fixation and precipitin tests, but that they were unable to produce antibodies in animals by injecting these substances.

These substances do not give any of the ordinary chemical reactions for protein, such as the biuret, Hopkins-Cole, Millon and sulphosalicylic acid reactions. The authors called such substances "residue antigens," and suggest "the usefulness of the residue antigen for specific precipitation and complement-fixation reactions for routine purpose in laboratory investigations." Following this suggestion, work was undertaken for the purpose of adapting residue antigens to the standardization of antimeningococcus serum. The first step was to find whether different types of meningococci carry specific residue antigens. For this purpose, 4 rabbits were immunized with a single strain each, each strain representing 1 of 4 different types of meningococci. The rabbits received intravenous injections with the living cultures on 3 consecutive days; they then rested 3 or 4 days, when 3 injections were again given. After 5 series of such injections, the rabbits yielded satisfactory serums, and were bled from the heart on the 5th day after the last injection. According to the investigations of Butterfield² and of Mörch,³ the 5th day seems to be the most suitable for bleeding animals immunized with meningococci.

Preparation of Residue Antigen.—Blake bottles with glucose agar were inoculated with suspensions from agar slants and incubated for 24 hours. The growth was then washed off with salt solution (25 c.c. being used to each bottle) and shaken in a shaking machine for from 5 to 6 hours. After shaking, the gross particles were removed by centrifugation until an almost clear, opalescent, yellowish fluid was obtained. The fluid portion was then removed and precipitated by

Received for publication, July 21, 1924.

¹ Jour. Exper. Med., 1923, 37, p. 75.

² U. S. Hygienic Lab. Bull. No. 124, 1920.

³ Rept. on Serological Investigations, etc., of League of Nations.

adding 10% acetic acid, drop by drop, until the precipitate came down. Usually little precipitate was obtained. This precipitate was removed by centrifugation, and supernatant fluid was boiled for from 2 to 3 minutes. The precipitate was removed again; then the hydrogen-ion concentration was adjusted to about P_H 7 by adding 10% NaOH. The fluid was then precipitated with 5 volumes of absolute alcohol and left at room temperature for from 12 to 16 hours until the precipitate came down; then it was centrifugalized, washed with alcohol and ether, and dried. The precipitate obtained was grayish-white and very small in amount; for instance, from 12 bottles of culture there was usually obtained from 0.18 to 0.25 gm. of material.

Various methods of preparing antigen were tried, but the most satisfactory is that just described.

TABLE 1
THE PRECIPITIN TEST OF MONOVALENT RABBIT ANTIMENINGOCOCCUS SERUM WITH
RESIDUE ANTIGENS

Residue Antigens	Antimeningococcus Serum			
	For Type 1	For Type 2	For Type 3	For Type 4
Type 1.....	10,000	500	0	500
Type 2.....	1,000	20,000	0	0
Type 3.....	500	0	1,000	0
Type 4.....	0	500	0	5,000

The figures give the highest dilutions of antigens giving distinct precipitate; 0 means no reaction.

Dilution of Residue Antigen.—Antigen in order to be dissolved was ground with a small amount of distilled water and diluted to the desired concentration with the same fluid. If an insoluble residue appeared, a few drops of acetic acid were added, and after dilution the hydrogen-ion concentration was adjusted to P_H 7. 0.01 gr. antigen was dissolved in 1 c.c. distilled water. This dilution was taken as dilution 1:100.

The Precipitin Test.—Antigens prepared from the 4 strains were tested with each serum. For the precipitin test, the whole serum was used by the contact or ring method. The final reading was made after 3 hours at room temperature. All antigens gave perfect control tests, i. e., no precipitation with normal rabbit serum. The serum of type 2 showed the highest titer, and the serum of type 3 showed the lowest titer (table 1).

The Complement-fixation Tests.—The complement-fixation tests were made in accordance with a method used in the Hygienic Laboratory, and with residue antigens as well as suspensions of meningococci. The

comparison showed higher titers of the serums with suspensions of meningococci than with residue antigens. However, the serums which gave low titers with suspensions of meningococci gave comparatively low titer with residue antigen and vice versa (tables 2 and 3).

The results of the complement-fixation and precipitin tests run parallel. The serums that gave cross precipitation in low dilution of residue antigen gave also in low dilutions a cross complement-fixation;

TABLE 2
COMPLEMENT-FIXATION OF MONOVALENT RABBIT ANTIMENINGOCOCCUS SERUM WITH
RESIDUE ANTIGENS

Residue Antigens	Antimeningococcus Serum			
	For Type 1	For Type 2	For Type 3	For Type 4
Type 1.....	80	10	0	5
Type 2.....	10	320-3	10	0
Type 3.....	0	0	40	0
Type 4.....	0	0	0	80

The tests were made with dilutions of residue antigens 1:1,000. The figures give the highest dilution of the antiserum, giving definite complement-fixation; 0 means no reaction.

TABLE 3
COMPLEMENT-FIXATION OF MONOVALENT RABBIT ANTIMENINGOCOCCUS SERUM WITH
SUSPENSIONS OF MENINGOCOCCI

Suspensions of Meningococci	Antimeningococcus Serum			
	For Type 1	For Type 2	For Type 3	For Type 4
Type 1.....	320	0	0	0
Type 2.....	0	640	0	0
Type 3.....	5	0	80	0
Type 4.....	0	0	0	160

The figures give the highest dilution of antiserum with which definite fixation of complement was obtained; 0 means no reaction.

for instance, serum of type 2 gave precipitation with residue antigen of type 1 and also complement-fixation in low dilution of the antiserum.

SUMMARY

The complement-fixation and precipitin tests indicate that the residue antigens obtained from each type of meningococci give specific reactions with homologous serums. The heterologous serums give either feeble cross reactions or none at all.

This work, while on too small a scale to permit definite conclusions, seems to indicate that the residue antigens from different types of meningococci are specific and distinct.

Investigations of the adaptability of residue antigen for the standardization of antimeningococcus serum will be continued.

PRACTICAL RESULTS WITH A FLOCCULATION TEST FOR SYPHILIS

C. B. MCG LUMPHY, M.D.

From the Pathological Laboratory of Northwestern University Medical School, Chicago

A flocculation test for the serum diagnosis of syphilis has been described by me, with the results in a series of 400 cases.¹ With this method there was agreement with the Wassermann reaction in approximately 98% of the serums studied; furthermore, a small number of cases which were diagnosed as syphilis, but which gave a negative Wassermann reaction, were positive with the flocculation test. The results obtained in this series encouraged further study for determining the practical value of this method.

Serums have been obtained from several laboratories, and the Wassermann tests were made by 5 persons. The latter fact is important, since it has reduced the personal element to a general average. Tests which were not in agreement with the results of the Wassermann reaction were repeated when serum was available and, in addition to testing with a different extract of human heart, tests were made with extracts from beef and horse hearts. In no case did a serum which was negative with human heart extract give a positive reaction with extracts from beef or horse heart. In addition, a series of 300 serums was tested with these 3 different extracts, and there was full agreement as to results. The choice of the heart tissue to be used is apparently a question of convenience, since the active substance is perhaps of the same nature in all.

The method of preparing the antigen and of performing the test has not been changed from that described in the previous paper. It may be emphasized that glycerol is added to the alcoholic extract and the 2 reagents well mixed before the final dilution with 3% sodium chloride solution. The amount of glycerol added to the alcoholic extract is that quantity that will furnish from 0.05 to 0.1 c.c. for each test or from 5 to 10% of the mixture of serum, glycerol and 3% sodium chloride solution. In addition to increasing the size of the floccules in positive serums, the glycerol gives a transparent mixture. Such a mixture to

Received for publication, July 24, 1924.

¹ Jour. Lab. & Clin. Med., 1924, 8, p. 539.

which clear serum has been added is distinguished from distilled water only by the slight color of the serum. This solution, which shows no appreciable change when the reaction is negative, furnishes an ideal menstruum for the recognition of the suspended floccules which form when the reaction is positive. The method of preparing the alcoholic extract and making the test will be described.

Preparation of Alcoholic Extract.—An extract is prepared from heart muscle (human, beef or horse) in the following manner: The muscle after being freed from fat and fibrous tissue is hashed and thoroughly dried at incubator temperature, after which it is reduced to a fine powder. The powder may be extracted at once with ether, or a first extraction made with acetone, which is perhaps to be preferred. One part of the powder is extracted for 2 or 3 days at incubator temperature with 9 parts acetone. The acetone is then poured off and the powder kept at 37 C. until the odor of acetone has disappeared. In this state, the powder will keep for months and probably indefinitely without any change. The extraction with ether may be carried out at room temperature, and should be repeated 2 or 3 times at intervals of 12 hours or longer. The container should be well shaken several times during the process of extraction. Approximately 1 gm. of the powder is extracted with 9 c.c. of ether. The ether used for the last two washings may be filtered and used again for the first two washings when preparing another extract. After the last ether has been poured off, the powder is allowed to dry until no odor of ether can be recognized. Without removing the ether insoluble residue from its container, 95% alcohol is added in the proportion of 9 c.c. to each gram of powder which has been extracted with ether. This mixture is allowed to remain at incubator temperature for 3 or 4 days, during which time it is well shaken several times daily. The alcoholic solution is then filtered, and the filtrate tested for its flocculating properties with several known positive and negative serums.

Standardization of the Alcoholic Extract.—An alcoholic extract which always produces flocculation when added to syphilitic serum and, on the other hand, does not flocculate in the presence of negative serum, may be regarded as ideal. Such an extract will probably not be obtained, but repeated observations indicate that practically all extracts prepared as described will produce flocculation in more than 90% of serums from persons giving a positive Wassermann or with active symptoms of syphilis.

In the Meinicke method,² the alcoholic extract is tested, and, if necessary, diluted with alcohol, while in the Kahn method,³ the alcoholic extract is the constant and salt solution the variable factor. Whether a method of standardization is of value could be determined only by testing a large number of positive and negative serums with varying amounts of alcoholic extract. With a highly concentrated extract and

² München. med. Wchnschr., 1919, 33.

³ Am. Jour. Pub. Health, 1924, 6, p. 498.

a dilute extract, such a procedure should show variation in the results, and necessarily a number of false positives, or false negatives, or doubtful reactions.

In order to determine the necessity of standardizing the alcoholic extract when prepared as described, the following experiment was made: An alcoholic extract was prepared in the regular manner, except that the proportion was 1 gm. of powdered heart to 4.5 c.c. of 95% alcohol. This mixture of several dried and powdered hearts when extracted with ether and finally with 95% alcohol repeatedly yielded an extract that was satisfactory when 0.1 c.c. was used for each test. After extracting for 4 days at 37 C., the alcoholic solution was filtered and the residue again extracted with 95% alcohol at incubator temperature. This second extract when tested with known flocculating serums showed no flocculation, thus proving that the active substance had been entirely removed by the first extraction with alcohol.

A series of 416 serums was tested with this concentrated alcoholic extract: For each serum, 3 antigens were prepared by the addition of glycerol and 3% sodium chloride solution in the regular manner except that the amount of alcoholic extract used was the amount that would furnish 0.1 c.c., 0.05 c.c., and 0.025 c.c. for each test. That the alcoholic content would be the same in all 3 solutions, 0.05 c.c. and 0.075 c.c. of alcohol for each test were added to the last 2 solutions.

It was expected that certain negative serums when tested with a highly concentrated antigen would show flocculation which, however, would not occur with an antigen one-fourth or one-half as strong. In this series of 416 serums, 36 gave a positive and 380 a negative Wassermann reaction, and the flocculation method checked in all but 14 cases.

In the series tested with extracts of different concentration, 14 serums gave a positive Wassermann and a negative flocculation reaction; 4 of these serums were from cases diagnosed clinically as syphilis, in 4 no diagnosis was stated, while in 6 cases syphilis was apparently excluded. Of special interest are the results obtained with antigens of different strength. It is to be noted that in only 3 serums were different results obtained with the weakest and the strongest extracts, while in 380 negative serums all flocculation tests were in agreement.

From these results it may be inferred that weak positive cases might be missed when a weak antigen was used, but, on the other hand, and doubtless of greater importance if any single test is to be relied on to determine a diagnosis, false positive flocculation reactions do not occur when a highly concentrated extract is used as antigen.

Numerous specimens of human heart tissue have been extracted and tested. With the exception of one specimen, all have been found to be satisfactory when 0.1 c.c. of the alcoholic extract was used for each test; therefore, this empirical quantity may be adhered to in general. An actual trial with a number of known positive and negative serums will at once determine whether the extract is suitable for use.

Dilution of Alcoholic Extract.—The alcoholic extract is diluted immediately before adding it to the serums to be tested. If 0.1 c.c. of the extract has been found to be satisfactory when tested with serums whose reaction with the flocculation method are known, this amount will be the unit in calculating the quantity to be used in a series of tests. For each serum, from 0.05 c.c. to 0.1 c.c. of glycerol are added to the alcoholic extract, and the two reagents are thoroughly mixed. To this mixture sufficient 3% sodium chloride solution

is added to furnish from 0.65 to 0.6 c.c. for each serum, and the mixture is well shaken. Spontaneous flocculation does not occur. A control tube containing the diluted extract shows no flocculation after 72 hours at 37 C.

The Test Proper.—From 0.2 to 0.3 serum should be used for the test and markedly hemoglobin-stained serums avoided. It is true that many positive reactions have been obtained with hemoglobin-stained serums from syphilitic cases, but the results are not always satisfactory. The serums having been transferred to small clean tubes, 0.8 c.c. of the diluted extract are added, after which the tubes are well shaken and placed in the incubator, which should not vary more than 2 or 3 degrees from 37 C. Most serums which give a positive reaction are recognized as such after 12 to 18 hours' incubation, but a second reading after 36 to 48 hours is advisable, since the reaction is then more marked, and certain serums which may have been doubtful at the end of the 18 hour period are readily recognized as positive at the second examination. When reading results, the tubes should be held near an electric light with a dark background, and the observer should look directly through the solution into the light.

Strong positive serums show pinhead-sized floccules and others of smaller dimensions which can be recognized with the naked eye, and often without the aid of artificial light and a dark background. As a routine method of examination, a small hand lens is recommended, since weak positives are then more readily recognized. The contents of certain negative tubes may show a diffuse granular appearance which is likely to be confusing to one not familiar with flocculation methods, but as a rule the negative specimens show no change. A positive serum shows definite floccules, and with some experience such are readily distinguished from negative tubes whose contents present a granular appearance. Doubtful cases may also be examined microscopically with low magnification. The floccules then appear as clumps which are scarcely to be distinguished from clumps of agglutinated bacteria.

In classifying results, no effort has been made to group positive serums according to a numerical classification as in the Wassermann test, but instead positive results have been designated, weak, plain, and strong. A weak reaction is one which by an experienced observer would be designated suspicious when seen with the naked eye and readily recognized as positive when examined with a lens giving slight magnification. Plain positives are readily recognized macroscopically, while strong positives are characterized by the presence of floccules which may attain 2 mm. in their greatest diameter in addition to others of smaller size. There is not constant agreement in the strength of the Wassermann and the flocculation reactions. In this series, certain serums which gave a +++ Wassermann reaction gave a weak positive flocculation reaction. Again, some serums with +++ and ++ Wassermann reactions gave strong flocculation reactions. Furthermore, the time element must be considered, as many positive tubes show a more marked reaction after a period of 36 hours' incubation, and although designated weak after 18 hours' incubation, are readily recognized as strong positive reactions when again observed after from 36 to 48 hours' incubation.

Bacterial Contamination.—Serums which are not more than two days old will rarely show contamination after 36 hours' incubation, and a less number after 18 hours' incubation, at which time the results are usually noted. This is probably to be explained by the fact that for a period of from 18 to 36 hours, the percentage of alcohol in the mixture of serum and antigen is sufficient to inhibit bacterial growth, but the rapid evaporation of alcohol which occurs at incubator temperature gradually decreases the antiseptic properties of the

mixture to such degree that multiplication of bacteria finally begins. While the presence of alcohol in the mixture serves a useful purpose in that it inhibits the growth of bacteria, alcohol to some extent interferes with the reaction. This has been demonstrated by making comparative tests in duplicate and tightly plugging one set of tubes with cork stoppers. The uncorked specimens which give a positive reaction show much larger floccules, while the reaction occurs sooner and is more marked than is the case with the corresponding tubes which have been plugged.

Wassermann Method.—With the exception of a small number of cases which were tested by a Wassermann method in which the antihuman hemolytic system was used, the antisheep hemolytic system was the choice of the laboratories which furnished the serums studied. Acetone-insoluble extracts of beef heart, one with and one without the addition of cholesterin, served as antigens.

TABLE 1
RESULTS WITH 1,836 SERUMS

	No.	Percentage
Wassermann negative, flocculation negative.....	1,627	88.61
Wassermann positive, flocculation positive.....	150	8.17
Total number of tests in agreement.....	1,777	96.78
Wassermann positive, flocculation negative.....	50	2.72
Wassermann negative, flocculation positive.....	9	0.49
Total number of tests not in agreement.....	59	3.21

TABLE 2
POSITIVE TESTS IN AGREEMENT

No.	Wassermann Test	Flocculation Test	Clinical Diagnosis
77	+ + + +	+	Syphilis
8	+ + + +	+	Syphilis
49	+ + + +	+	No diagnosis
4	+ + + +	+	No diagnosis
1	+ + + +	+	No diagnosis
10	+ + + +	+	Other than syphilis (see table 3)
1	+ + + +	+	Other than syphilis (see table 3)
Total 150			

Antigen, serum and complement were mixed and incubated at 37 C. for one-half hour. The amboceptor and sheep cells were then added and incubation again carried out for a period of one-half hour. The results were then read.

The results obtained with the Wassermann reaction and the flocculation method are set forth in the tables.

Table 1 includes the serums that gave + + + or a + + Wassermann reaction as well as those which gave a + + + + Wassermann reaction. Furthermore, the figures indicate percentage of agreement without regard to the clinical diagnosis.

Three serums which were anticomplementary and which gave a negative flocculation reaction have not been included since clinical data could not be obtained.

In table 2 are included all serums which gave a positive reaction with both the Wassermann and flocculation tests. No diagnosis can be given in a considerable number of this group since clinical data could not be obtained. In addition, a small percentage gave false positive results with both the Wassermann and flocculation tests.

Table 3 includes a group of cases in which the clinical diagnosis was stated to be other than syphilis. Since the Wassermann and flocculation tests were in agreement, the results must be classified as unspecific reactions. However, since the laboratory findings are strongly suggestive of syphilis, the possibility of error in the clinical diagnosis is to be considered.

TABLE 3
POSITIVE TESTS IN AGREEMENT — APPARENTLY UNSPECIFIC REACTIONS

Wassermann	Flocculation	Clinical Diagnosis
++++	+	Chronic fibrous pleurisy
++++	+	Arthritis
++++	+	Gonorrhea
++++	+	Tachycardia
++++	+	Eczema
++++	+	Mass in abdomen
++++	+	Abscess
++++	+	Chronic pleurisy
++++	+	Acute arthritis
++++	+	Ulcer
++++	+	Chronic interstitial nephritis

TABLE 4
REACTIONS NOT IN AGREEMENT

No.	Wassermann Test	Flocculation Test	Clinical Diagnosis
1	++++	—	Syphilis; treated
2	++++	—	Syphilis
3	++++	—	Syphilis; arthritis
4	++++	—	Tuberculosis; antisyphilitic treatment
5	++++	—	Syphilis
6	++++	—	Syphilitic ulcer
7	++++	—	Acne; antisyphilitic treatment
8	++++	—	Syphilis
9	—	+	Syphilis
10	—	+	Syphilis
11	—	+	Syphilis; cardiac decompensation

TABLE 5
REACTIONS NOT IN AGREEMENT — CLINICAL DIAGNOSIS OTHER THAN SYPHILIS

No.	Wassermann Test	Flocculation Test	Clinical Diagnosis
1	++++	—	Pregnancy
2	++++	—	Pregnancy
3	++++	—	Pregnancy(?); leukorrhea
4	++++	—	Pyelitis
5	++++	—	No diagnosis
6	++++	—	No diagnosis
7	++++	—	No diagnosis
8	—	+	Dead fetus in utero
9	—	+	No diagnosis
10	—	+	No diagnosis
11	—	+	Subacute arthritis (?)
12	—	+	No diagnosis; repeated flocculation negative
13	—	+	No diagnosis

Table 4 shows that 11 serums from cases which were recognized clinically as syphilis gave different results with the Wassermann and the flocculation methods. Serums 4 and 7 have been included because antisyphilitic treatment was prescribed in these cases. Eight serums which were positive with the Wassermann test gave negative results when tested with the flocculation method,

while only 3 serums gave a negative Wassermann and a positive flocculation reaction.

These results indicate that more positive cases would be detected by means of the Wassermann than by the flocculation method. On the other hand, a small number of serums which gave a negative Wassermann gave a positive flocculation test, and in such cases, especially if clinical findings are suggestive of syphilis, a positive flocculation test would be of value in making a diagnosis. A negative flocculation test apparently should not exclude a diagnosis of syphilis when the Wassermann test was positive, while a negative Wassermann and a positive flocculation should call for a further study of the serum and a

TABLE 6
REACTIONS NOT IN AGREEMENT

No.	Wassermann Test	Flocculation Test	Clinical Diagnosis
1	+++	—	Syphilis; treated
2	+++	—	Syphilis
3	+++	—	Syphilis; treated
4	+++	—	Myalgia
5	+++	—	Fibroid
6	+++	—	Alcoholic optic neuritis
7	+++	—	Edema of prepuce
8	+++	—	Spastic colitis
9	+++	—	No diagnosis
10	++	—	No diagnosis
11	+++	—	Syphilis
12	+++	—	Syphilis
13	+++	—	Syphilitic granuloma
14	+++	—	Syphilis; treated
15	+++	—	Syphilitic ulcer
16	+++	—	Fibrolipoma
17	+++	—	Asthma; bronchitis
18	+++	—	Chancroid
19	+++	—	Pleuritis
20	+++	—	Arthritis; repeated Wassermann negative
21	+++	—	Chronic bursitis; tuberculosis
22	+++	—	Epilepsy; repeated Wassermann negative
23	+++	—	Myalgia
24	+++	—	Tracheobronchitis
25	+++	—	Chronic constipation; repeated Wasserman tests negative
26	+++	—	Perforated septum
27	+++	—	Gastric ulcer
28	+++	—	Enlarged prostate
29	+++	—	Arteriosclerosis
30	+++	—	Fibroid
31	+++	—	Tuberculous sinus
32	+++	—	Pregnancy; repeated Wassermann test negative
33	+++	—	No diagnosis
34	+++	—	No diagnosis
35	+++	—	No diagnosis; repeated Wassermann tests negative

careful clinical examination before definitely ruling out the possibility of syphilitic infection.

Of the thirteen cases in table 5, it is seen that +++ Wassermann reactions were obtained in cases in which syphilis may be excluded. No diagnosis was made in the 3 remaining cases which gave a positive Wassermann and a negative flocculation reaction. Of the six cases which gave a positive flocculation and a negative Wassermann test, no diagnosis was obtained in 4; one case was diagnosed as subacute arthritis (?) and another as "dead fetus in utero." Since so many cases in this group were not diagnosed, no definite statement can be made except that several +++ Wassermann reactions were obtained in apparently nonsyphilitic cases.

In table 6 are grouped a series of cases which include serums which gave +++ Wassermann reactions and a larger number which gave ++ Wasser-

mann reactions. Three serums which gave a +++ Wassermann and a negative flocculation reaction were diagnosed clinically as syphilitic, while five cases of syphilis gave a ++ Wassermann and a negative flocculation reaction. Three of these positive cases had been treated. In addition to the 8 cases diagnosed clinically as syphilis, there were 27 cases to be considered. Five cases in which no diagnosis was stated gave a positive Wassermann reaction (one serum was negative 3 days later) and a negative flocculation test. The remaining 22 cases in which the Wassermann reaction was positive and the flocculation test negative are instructive, since in this group the clinical diagnosis was definitely stated. It is important to note that in 5 of these cases repeated Wassermann tests were negative. From an analysis of this group, it is evident that many false positives were obtained with the Wassermann reaction, while the results with the flocculation method are more in harmony with the clinical findings.

SUMMARY

The value of the Wassermann reaction as an aid in the diagnosis of syphilis is so well recognized that it is customary and proper to compare other methods of laboratory diagnosis with this well-known test. At the same time, it must be recognized that the clinical evidence revealed by critical examination is the final arbiter, and must be given the greatest weight in deciding the diagnosis.

In attempting to correlate clinical and laboratory findings, the usual difficulties have been encountered. A considerable number of cases were not diagnosed clinically for the reason that most of the serums studied were obtained from dispensary patients, and many of these did not return after blood was taken for examination. In many cases, clinical data could not be obtained for different reasons. However, it is believed that enough clinical data are at hand to allow certain conclusions to be drawn.

Of chief interest is the high percentage of agreement of the two tests. This is perhaps only slightly less than is to be expected with the results obtained by different Wassermann laboratories. Next in importance is the fact that a number of serums which gave a positive Wassermann test were negative when tested by the flocculation method. Since a diagnosis of syphilis was made in a number of these cases, the Wassermann test was the more sensitive of the two reactions. On the other hand, a larger number of false positives were obtained with the Wassermann method which shows the necessity of repeating this test, especially if clinical signs do not suggest syphilis.

The flocculation method here presented is not offered as a substitute for the Wassermann reaction. However, it can be carried out at the same time the Wassermann test is made and, because of its simplicity,

the additional time required is a negligible factor. Since the Wassermann test has been made in millions of cases and, in spite of numerous modifications and doubtless often poor technic, has demonstrated its value, no test should be substituted which has not been subjected to the most critical investigation. Beyond question, certain syphilitic serums are positive when tested by the flocculation method and negative when tested by the Wassermann method. Such results with the flocculation test are of value to the clinician since there is an additional factor which is to be considered in the diagnosis.

CONCLUSIONS

The results of the Wassermann and the flocculation tests agree in more than 95% of serums.

The Wassermann test is more sensitive than the flocculation method, but it gives more false positive reactions.

A small percentage of syphilitic serums give a negative Wassermann and a positive flocculation reaction.

False positive flocculation reactions do not occur when a highly concentrated antigen is added to nonsyphilitic serums.

The results obtained with antigens prepared from human, beef, and horse heart are in agreement.

IMMUNOLOGIC SIGNIFICANCE OF VITAMINS

V. RESISTANCE OF THE AVITAMINIC ALBINO RAT TO DIPHTHERIA TOXIN; PRODUCTION OF ANTITOXIN AND BLOOD PRESSURE EFFECTS

C. H. WERKMAN, F. M. BALDWIN AND V. E. NELSON

From the Departments of Bacteriology, Zoology, and Chemistry, Iowa State College, Ames

No adequate explanation has been offered to account for the greater susceptibility of avitaminic animals to bacterial infection although the rupture in resistance is definite and determinable. Among the explanations that have been offered are: (1) the presence of "degenerative changes and feeble leukoblastic reaction seen in the bone marrow of chronic scurvy guinea-pigs;¹ (2) inability to produce antibodies (agglutinins);² (3) reduced bactericidal activity of the blood serum;³ (4) lowered body temperature and concomitant effects, i. e., depressed phagocytosis,⁴ better growth conditions for the micro-organism.⁵ From the work done, it is apparent that several factors are of undoubted importance in accounting for the failure of animals deficient in vitamin to resist infection, as well as normal healthy individuals, and that resistance is not wholly dependent on any one factor.

The present paper is a study of the effect of diphtheria toxin on avitaminic albino rats; their resistance to toxin injections, their ability to react by the production of antitoxin, and the effect of the toxin on the blood pressure of avitaminic B rats and normal controls.

Although a vitamin deficiency in an animal may lead to infection, it is quite conceivable that the effect may be the result of superimposing the injurious effects of a small relatively unimportant infection on the injurious vitamin deficiency effects, resulting in a depressed metabolism that prevents the animal from exerting a normal effort to overcome the infection or toxemia. This we shall see may be particularly true for toxin effects.

Toxin experimentally injected into B-minus and normal control rats may exert quantitatively the same effect, but in the case of the

Received for publication, July 26, 1924.

¹ Findlay: Jour. Path. & Bacteriol., 1923, 26, p. 1.

² Guerrini: Ann. d'Ig., 1921, 31, p. 596.

³ Smith and Wason: Jour. Immunol., 1923, 8, p. 195.

⁴ Werkman: Jour. Infect. Dis., 1923, 32, p. 263.

⁵ Werkman: Ibid., 1924, 34, p. 447.

animals deficient in vitamin the effect may be superimposed on that already produced as the result of vitamin deficiency. Such a summation of depressions of physiologic activities would then result in a seriously reduced metabolism. Either injury acting alone would not prove fatal, assuming the degree or intensity of the injury not to increase. Of course, both "injury loads" may be increasing and either alone given time produce death. It is readily possible to produce in animals a vitamin deficiency which when added to a definite toxin effect or "load" results in death although the same "toxin load" is well borne by normal healthy animals. The "toxin load" is a function of toxin intensity (dosage) and time; similarly, with "vitamin deficiency load."

In case one or both "loads" is progressing to a fatal termination and both are present, the critical minimum in the depressed metabolism is more quickly reached, and death results earlier. This condition will be exemplified in the work on blood pressure of normal and avitaminic rats injected with diphtheria toxin.

EXPERIMENTS

Month old albino rats were used in these experiments. The basal ration consisted of casein (alcohol extracted) 18%, dextrin 74%, McCollum's salt mixture 3%. Yeast (3%) or filtered butterfat (3%) supplied vitamin B or A as required by the experiment.

The diphtheria toxin used in this work possessed an MLD of 0.0027 c.c. and an L⁺ of 0.198 c.c. At the time of use, it had been aged for 2 years. The toxin was furnished by Dr. W. H. Park of the New York Department of Health.

Resistance of the Avitaminic Albino Rat to Injection of Diphtheria Toxin.—Coca, Russel, and Baughman,⁶ working with mature rats weighing almost 300 gm., found that 4,000 MLD regularly proved fatal, although 1,000 MLD were usually successfully resisted. Since young rats were employed in the present studies, the minimal lethal dose was less than that found by the previous investigators when considered from the standpoint of units of toxin required to kill, although comparable when considered as units of toxin per gram of rat.

Table 1 summarizes the resistance of normal and avitaminic albino rats to diphtheria toxin. These results were substantiated by several similar experiments using diphtheria and botulinus toxins. Difficulty in maintaining a fixed toxicity of the botulinus toxin led to less consistent results than were obtained with the diphtheria toxin. The results in table 1 were compiled from two similar experiments, which accounts for the two distinct weights of the animals. In general, the greater the degree of vitamin deficiency, the greater the susceptibility to infection or toxemia. This may be seen in the case of animals 29 and 30. Both rats received the same B-deficient ration, but, due to the maturity of the heavier animal, it did not suffer the effects of the vitamin deficiency to the extent that the younger, more actively growing animal did, and therefore successfully resisted an injection of toxin not withstood by the younger rat in urgent need of the vitamin.

⁶ Jour. Immunol., 1921, 6, p. 387.

Ordinarily rats will not tolerate injections of toxin directly proportional to their body weights. With increase in weight, the toxin units per gram of rat required to kill decreases somewhat. However, in the case of the heavier rat mentioned above, its survival is due, in all probability, to the fact that it was in relatively good condition. This is indicated by its rectal temperature.

TABLE 1
RESISTANCE OF THE AVITAMINIC ALBINO RAT TO DIPHTHERIA TOXIN

Rat No.	Treatment	Weight in Gm.	Rectal Temperature, F.	Dosage per 10 Gm. of Rat, C c.	Dose, C c.	Toxin Units per 10 Gm. of Rat	Results
1	Control	110	101.6	0.050	0.55	18.5	Lived
2		85	102.1	0.050	0.43	18.7	Lived
3		80	101.7	0.075	0.60	27.8	Lived
4		80	101.5	0.100	0.80	29.6	Lived
5		50	99.9	0.125	0.63	46.6	Lived
6		135	102.1	0.150	2.04	55.7	Died after 135 hours
7		85	100.4	0.175	1.49	64.9	Lived
8		75	100.8	0.175	1.31	64.7	Lived, paralysis
9		80	101.9	0.175	1.40	64.9	Lived, paralysis
10		80	101.4	0.200	1.60	74.1	Died after 120 hours
11		70	102.3	0.230	1.61	84.7	Died after 72 hours
12		130	101.4	0.300	3.90	111.1	Died after 45 hours
13		110	100.8	0.400	4.40	148.1	Died after 30 hours
14		120	102.8	0.500	6.00	185.2	Died after 30 hours
15	A minus	80	101.1	0.050	0.40	18.5	Lived
16		65	101.4	0.100	0.65	29.6	Died after 105 hours
17		55	102.1	0.125	0.69	46.3	Died after 54 hours
18		90	100.7	0.150	1.35	55.5	Died after 56 hours
19		80	99.3	0.175	1.40	64.8	Died after 48 hours
20		65	99.7	0.175	1.14	64.8	Died after 50 hours
21		60	97.0	0.200	1.20	74.1	Died after 24 hours
22		80	100.0	0.230	1.84	85.2	Died after 38 hours
23		140	100.2	0.300	4.20	111.1	Died after 33 hours
24		105	99.0	0.400	4.20	148.1	Died after 30 hours
25		120	100.0	0.500	6.00	185.2	Died after 32 hours
26	B minus	110	99.4	0.050	0.55	18.5	Lived
27		90	94.0	0.050	0.45	18.5	Lived
28		120	99.6	0.073	0.88	27.3	Died after 96 hours
29		65	93.7	0.100	0.65	29.6	Died after 74 hours
30		110	100.8	0.160	1.10	29.6	Lived, paralysis
31		45	99.6	0.125	0.56	46.1	Died after 48 hours
32		55	97.0	0.150	0.83	55.8	Died after 45 hours
33		130	100.3	0.150	1.95	55.5	Died after 64 hours
34		90	100.7	0.175	1.58	65.0	Died after 36 hours
35		60	94.3	0.175	1.05	64.8	Died after 30 hours
36		40	99.5	0.200	0.80	74.1	Died after 30 hours

MLD (250 gm. guinea-pig) = 0.0027 c c.

The A-deficient rats do not show the even progressive drop in resistance manifested by the B-deficient rats. Their break in resistance is more sudden, corresponding to the suddenness with which symptoms of A-deficiency often show up.

Calculated on the basis of units of toxin per 10 gm. of rat necessary to produce the death of the animal, there were required approximately 30 units for the A-minus; a similar number (28) of units for the B-minus, whereas the normal rats required from 56 to 75 units.

These results might lead one to believe that the result is due to an actual rupture in the immunity mechanism of the rat. It is readily possible that

the cells of avitaminic rats absorb more toxin than the cells of normal animals. The cells of the normal rat are quite impermeable or do not attach to diphtheria toxin, and the poison continues to float in the blood stream. If cells of avitaminic rats were permeable to the toxin or the toxin attached itself more readily to the cells of avitaminic animals, the toxin would be removed from circulation. This point was determined, however, and the toxin was not found to be removed from circulation, testing the content of toxin in the serums on guinea-pigs. The permeability of the cells for the toxin was not determinably increased, since the toxin was found floating unattached for hours in the serum after injection. The avitaminic rats are not, then, more susceptible to diphtheria toxin because of any increased absorption. The

TABLE 2
EFFECT OF THE LACK OF VITAMINS ON THE PRODUCTION OF DIPHTHERIA ANTITOXIN BY THE ALBINO RAT

Guinea-Pig No.	Weight, Gm.	Anti-toxin (in 2 c c.) C c.	Treatment of Antitoxin Rats	Toxin (L + in 1 c c.)	Results	Anti-toxin per C c. Units
9	240	0.8	Control (Mixed Serums from 4 Rats)	0.198	Died after 44 hours	0.80
11	230	0.9		0.198	Died after 40 hours	
13	255	1.0		0.198	Died after 46 hours	
14	255	1.05		0.198	Died after 55 hours	
15	245	1.1		0.198	Died after 64 hours	
16	250	1.15		0.198	Died after 3 days	
17	255	1.2		0.198	Died after 3 days	
18	250	1.25		0.198	Died after 101 hours	
19	245	1.3		0.198	Died after 107 hours	
20	255	1.4		0.198	Lived	
33	260	0.9	A-minus (Mixed Serums from 4 Rats)	0.198	Died after 54 hours	0.77
35	270	1.0		0.198	Died after 54 hours	
36	265	1.1		0.198	Died after 55 hours	
37	255	1.15		0.198	Died after 60 hours	
38	245	1.2		0.198	Died after 73 hours	
39	240	1.25		0.198	Died after 77 hours	
40	250	1.3		0.198	Died after 93 hours	
41	250	1.35		0.198	Lived	
45	235	1.0	B-minus (Mixed Serums from 4 Rats)	0.198	Died after 55 hours	0.83
47	250	1.15		0.198	Died after 86 hours	
48	245	1.20		0.198	Died after 103 hours	
49	260	1.25		0.198	Died after 6 days	
50	255	1.3		0.198	Lived	
51	245	1.35		0.198	Lived	

quantitative determination of the toxin content established no differences in the amounts absorbed by avitaminic and normal control rats. This will be referred to again when the effect of vitamin deficiency and diphtheria toxin injections on blood pressure of the albino rat is considered.

Effect of Vitamin Deficiency on the Production of Diphtheria Antitoxin by the Albino Rat.—The production of diphtheria antitoxin in the albino rat occurs in relatively small amounts. Coca, Russel, and Baughman⁹ have shown the production of approximately 0.4 unit per c c. to result after 4 injections of toxin. The effect of vitamin deficiencies shown in table 2, was determined in 3 series of 4 rats each, including A and B minus and normal animals. Each rat received 5 three day intraperitoneal injections of 0.2, 0.4, 0.6, 0.8 and 1.0 c c. of toxin, and it was bled by heart puncture on the 9th day after the last injection. The antitoxin content of the serums was determined on guinea-

pigs after heating for 30 min. at 56 C. Toxin-antitoxin mixtures were allowed to stand for 1 hour at room temperature before injections were made. Table 2 shows no significant variations in the antitoxin productions by avitaminic and normal rats. Confirmatory results were obtained by injecting arbitrarily a definite quantity of antitoxin from avitaminic and normal rats into a series of rats of equal weight, determining the quantity of toxin required to kill in 6 days. The amounts necessary did not vary more than 0.2 c.c. for the avitaminic and normal rats when as much as 7 c.c. were required to kill in 6 days.

Effect of Diphtheria Toxin and the Lack of Vitamin B on the Blood Pressure of Albino Rats.—Although the normal albino rat is resistant to

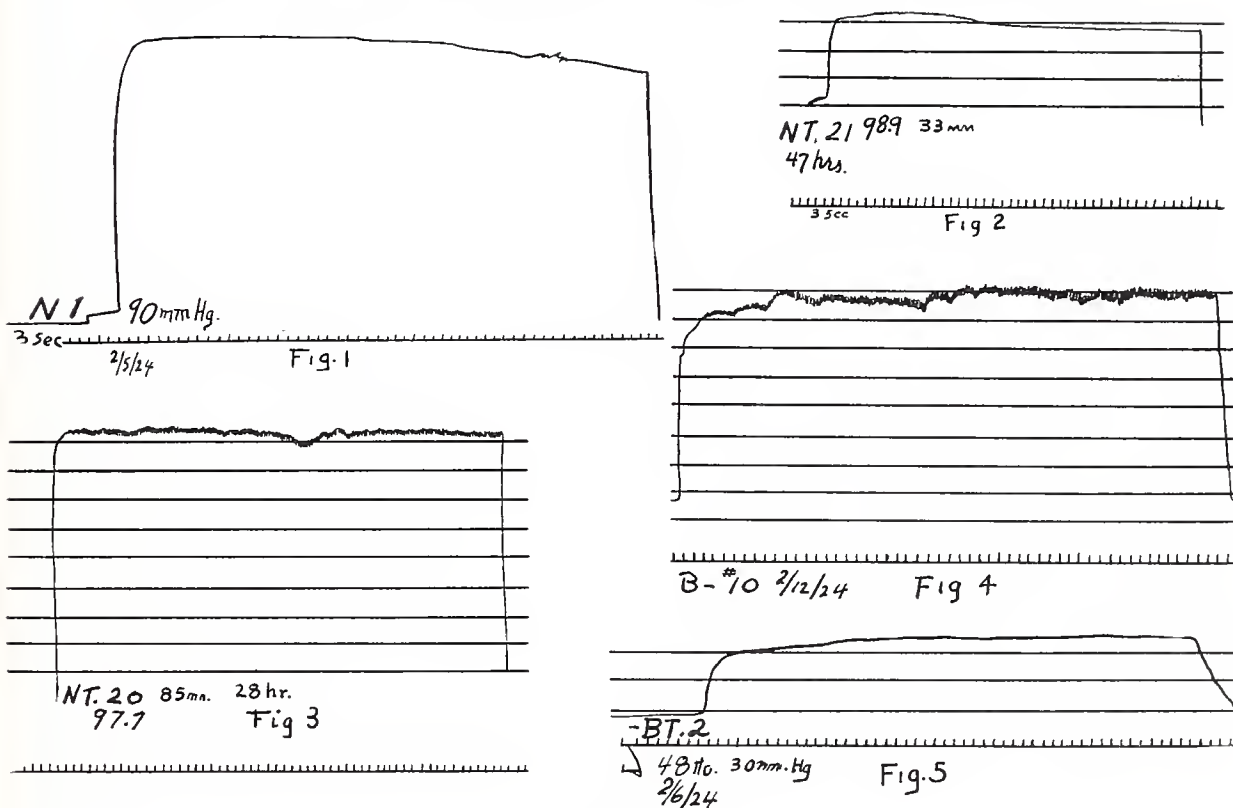


PLATE 1

Fig. 1.—Blood pressure of a normal rat, 90 mm. Hg.

Fig. 2.—Blood pressure of a normal rat 47 hours after lethal injection of diphtheria toxin, 33 mm. Hg.

Fig. 3.—Blood pressure of a normal rat 28 hours after lethal injection of diphtheria toxin, 85 mm. Hg.

Fig. 4.—Blood pressure of a rat lacking vitamin B six weeks on ration, 70 mm. Hg.

Fig. 5.—Blood pressure of a rat lacking vitamin B, six weeks on ration, 48 hours after lethal injection of diphtheria toxin, 30 mm. Hg.

enormous injections of diphtheria toxin, that the effect on the blood pressure is similar to that in man when injected in quantities sufficient to cause injury in the animal is shown by the kymograph records in figures 1 and 2 and by table 3. Fig. 1 shows normal pressure obtained from a 75 gm. rat by the

direct mercurial manometer method.⁷ Fig. 2 indicates the pressure in a 75 gm. rat 47 hrs. after the injection of a minimal lethal dose of diphtheria toxin for the rat. The rectal temperature of this rat was 98.9 F. Death would probably have resulted on about the 6th day. The drop in blood pressure following diphtheria toxin injection usually occurs on the second or third day after injection. The drop sometimes occurs on the 1st day, but may be delayed until the 4th day. It is often marked and occurs suddenly. Delay in the drop of blood pressure in diphtheria in man often occurs.⁸ Antitoxin administered in time inhibits the fall and prevents death. Meyers and Wallace⁹ have shown that the arterioles and capillaries in the splanchnic domain fail to react

TABLE 3
EFFECT OF DIPHTHERIA TOXIN ON THE BLOOD PRESSURE OF AVITAMINIC ALBINO RATS

Rat No.	Weight, Gm.	Treatment	Rectal Temperature (F.)			Blood Pressure in Mm. Hg		
			Before Injection	24-35 Hours after Injection	35-50 Hours after Injection	Before Injection	24-30 Hours after Injection	30-50 Hours after Injection
1	75	Normal Control	102.0	90
2	80		101.6	100
3	80		102.2	95
4	70		101.9	93
5	90		102.3	87
6	55	B-minus	97.3	60
7	60		99.7	70
8	65		93.8	37
9	70		94.6	48
10	75	Normal + toxin	100.7	103.1	98.8	77
11	75		101.0	102.4	98.9	33
12	75		100.2	96.2	85
13	70		101.8	102.9	97.7	47
14	75		102.6	103.3	95	..
15	70		101.5	98.9	85	..
16	60	B-minus + toxin	97.0	102.7	90.0	20
17	50		97.3	101.0	95.9	60
18	70		98.7	100.3	97.3	55
19	55		100.1	98.3	15
20	60		98.5	99.7	60	..
21	55		99.8	100.4	70	..

Toxin = calculated MLD for the animal in c.c.

normally to epinephrin and are engorged. Peripheral splanchnic paralysis may therefore account for the hypotension. The fall in pressure is generally proportional to the severity of the toxemia; it does not, however, bear any definite relationship to the intensity of the fever.

In Fig. 2, the animal showed a pressure of approximately 33 mm. Hg 47 hrs. after toxin was injected, and a rectal temperature of 98.9 F., whereas the pressure of one of its mates, shown in Fig. 3, 28 hrs. after injection showed a pressure of 85 mm. Hg and a rectal temperature of only 97.7 F.

The injection of toxin into the albino rat is followed by fever during the first day; the fever disappears and is followed by subnormal temperatures.

⁷ Baldwin, Cook and Nelson: *Am. Jour. Physiol.*, 1924, 68, p. 379.

⁸ Beck and Slapa: *Wien. klin. Wchnschr.*, 1895, 18, p. 323.

⁹ *Proc. Soc. Exper. Biol. and Med.*, 1914, 12, p. 43.

Subnormal temperature and low blood pressure may exist for several days before death intervenes.

Baldwin, Cook, and Nelson⁷ have reported subnormal pressures in B-minus rats. Fig. 4 indicates the blood pressure in a 50 gm. rat on a B-deficient ration for 6 weeks, showing an average pressure of about 70 mm. Hg. The drop due to B-deficiency is progressive and ranges from normal down to 15 mm. Hg before death.

The blood pressure of a B-deficient rat 48 hours after the injection of an MLD of toxin for the rat, is shown in fig. 5. The temperature of this rat was 96.4 F. A summary of results on blood pressures is given for a few typical animals in table 3. The drop in resistance of avitaminic rats to diphtheria toxin is the superimposing of deleterious effects rather than the break in the immunity mechanism; the effect of toxin injections, alike in normal and avitaminic animals, is, in the case of avitaminic animals, superimposed on that of vitamin deficiency.

The serious effects of vitamin deficiency on the myocardium have been pointed out by Baldwin, Cook, and Nelson;⁷ the injection of diphtheria toxin adds the myocardial lesions of diphtheria. Warthin,¹⁰ in a recent study of the myocardial lesions of diphtheria, has concluded that "the essential lesion of the heart in diphtheria is a toxic parenchymatous hyaline degeneration or necrosis, associated frequently with fatty degenerative infiltration and less frequently with cloudy swelling or a simple necrosis," and that the histologic picture will depend among other factors on "the associated nutritional conditions."

SUMMARY

Rats suffering from the lack of vitamins A or B succumb to the effects of smaller injections of diphtheria toxin than do normal animals on a complete diet. The susceptibility of the avitaminic animal is not due to any rupture of the immunity mechanism that normally serves to prevent an injurious action by the diphtheria toxin. The ability of the animals to produce antitoxin is not disturbed, and a small production of between 0.5 and 1.0 unit per c c. occurs after about 5 sublethal injections of toxin. The absorption of diphtheria toxin by the cells of B-minus rats is not greater than occurs in normal animals, and the toxin is found floating unattached for hours in the serum after injection.

The injection of diphtheria toxin into the the rat produces a marked and sudden drop in blood pressure, usually occurring on the second or third day after the injection is made. If a fatal dose of toxin has been injected, the drop is progressive to the point of death. The rat tolerates markedly low blood pressures and body temperatures several days before death intervenes.

¹⁰ Jour. Infect. Dis., 1924, 33, p. 32.

In agreement with the results of Baldwin, Cook and Nelson, the blood pressure of the rat suffers a progressive drop during vitamin B-deficiency proportional to the severity of the deficiency. The injection of diphtheria toxin into B-minus rats serves to hasten death, or results in a fatal termination when the additive effects of vitamin deficiency and toxin are sufficient to produce a depression of physiologic activities below a critical minimum.

THE GERMICIDAL PROPERTIES OF CHEMICALLY PURE SOAPS

JOHN E. WALKER

From the Laboratory Service, The Army and Navy General Hospital, Hot Springs, Ark.

Since Robert Koch¹ first found that soap solutions possessed germicidal properties, a considerable literature has grown up about the subject. Some authors have failed to agree with Koch's conclusions. It is difficult to evaluate much of this work, however, since only ordinary commercial soaps were usually employed, and often even the fat or oil saponified in its manufacture was not known, or at least not stated. The first systematic work with chemically pure compounds seems to be that of Reichenbach,² who investigated the germicidal activity of the potassium salts of acids of the acetic acid series and of several of the unsaturated acids. Using *B. coli* as the test organism, he found that potassium stearate, palmitate, and myristate were distinctly germicidal. The palmitate was the most efficient. On the other hand, he found that soap made from the unsaturated acids (oleic, erucic, linolic, and elaidic acids) were almost entirely inert. Lamar,³ however, found that soaps of the unsaturated acids were strongly bactericidal toward the pneumococcus and streptococcus, a result pointing to the existence of selective action for the different organisms.

The subject has considerable practical importance, for it would be advantageous from the standpoint of hygiene to know what soaps are germicidal. Dish-water has been held in part, at least, responsible for the spread of respiratory infections by Cumming.⁴ Nichols⁵ showed that if the dish-water contains 0.5% soap (yellow or brown bar soap), this danger could practically be eliminated as far as pneumococci and streptococci were concerned. He also confirmed Lamar's observation relating to the high germicidal activity of sodium oleate toward the pneumococcus and streptococcus, but found that sodium stearate in 0.5 % solution was without effect on these organisms.

Received for publication, Oct. 4, 1924.

¹ Mittheil. kaiserl. Gesundheitsamtes, 1881, 1, p. 271.

² Ztschr. f. Hyg. u. Infektionskr., 1908, 59, p. 296.

³ Jour. Exper. Med., 1911, 13, p. 380.

⁴ Am. Jour. Pub. Health, 1919, 9, p. 849; Mil. Surgeon, 1920, 46, p. 150.

⁵ Jour. Lab. & Clin. Med., 1920, 5, p. 502.

In addition to these considerations of hygiene, there are many indications that soaps may be of importance in other respects. Noguchi⁶ found that extracts of leukocytes were bactericidal, and that this was due chiefly to their content of the soaps of the higher saturated acids. He also showed that mixtures of soap and inactivated serum possessed many points in common with complement. Klotz⁷ has found soap in inflammatory foci. Lamar³ suggested that soaps possibly are related to immune processes, representative, perhaps, of definite chemical compounds concerned in the defense against micro-organisms, in addition to the already known defenses of the various kinds of antibodies. During the war, several authors—among whom may be mentioned Haycraft⁸—found soaps useful in the treatment of wounds. Reasoner⁹ has shown that soap solutions have a rapid solvent action on *Spirochaeta pallida*. These facts seemed to make worth while a reinvestigation of the subject in the effort to correlate the different opinions expressed in the literature.

PREPARATION OF SOAPS

The soaps were prepared by mixing equimolecular proportions of the pure fatty acids and normal sodium or potassium hydroxide and heating on the water bath. This is the method of preparation used by Fischer¹⁰ in his study of the colloidal chemistry of soaps. The sodium and potassium salts of the following fatty acids were prepared: butyric, valeric, caprylic, caproic, capric, lauric, myristic, palmitic, stearic, oleic, linolic, and linolenic (only the sodium salt of the last acid). The solutions were diluted to a concentration of N/5 or until they were fluid at ordinary temperature. These solutions were kept in well stoppered flasks. They were invariably found to be sterile.

Source of Material.—The fatty acids were all obtained from the Eastman Kodak Company with the following exceptions: linolic and linolenic acids, from C. A. F. Kahlbaum; oleic and stearic acids, from manufacturers who did not claim absolute chemical purity. A portion of the linolic acid was kindly supplied by Dr. Martin H. Fischer.

No systematic testing of these acids was carried out, although the following observations would indicate that most of them (with the exception of stearic acid) were of a high degree of chemical purity. Melting points: lauric, 42.5 C. myristic, 52; palmitic, 61.5; stearic, 56. Saponification numbers: lauric, 279 (theoretical, 280); oleic, 197 (theoretical, 199); linolic, 201 (theoretical, 200).

TECHNIC OF TESTS

In a general way, the method laid down by Anderson and McClintic¹¹ for the determination of phenol coefficients was followed, except that other

⁶ Biochem. Ztschr., 1907, 6, p. 327.

⁷ Jour. Exper. Med., 1906, 8, p. 322.

⁸ Brit. Med. Jour., 1918, 1, p. 80.

⁹ Jour. Am. Med. Assn., 1917, 68, p. 73.

¹⁰ Soaps and Proteins, 1921.

organisms in addition to *B. typhosus* were used, and that successive dilutions of the soaps differed in strength by one-half. Each test tube contained 5 c.c. of the soap dilution in distilled water, and each tube was inoculated with 0.1 c.c. of broth culture. After the time intervals ($2\frac{1}{2}$ and 15 minutes), a 4 mm. loopful was transferred to broth, which was incubated for 48 hours and then observed for growth, being plated out if the soaps transferred over caused any clouding. Control tests showed that in no instance was enough soap carried over to the broth to inhibit growth.

The tests, with the exceptions noted, were all carried out in a 20 degree water bath.

Phenol was used as a control germicide, 4 tubes (2%, 1%, 0.5%, and 0.25%) being run with all the tests. The phenol solution was carefully standardized. At the beginning of the work, it was planned to work out an accurate phenol coefficient for each soap as it affected each organism, but this was abandoned because of the difficulty of duplicating results (especially for the pneumococcus and streptococcus) with the soaps from day to day when the different dilutions lay as close together as advised by Anderson and McClintic. Much of this irregularity with the soaps disappeared, however, when scrupulous care was taken to avoid foaming in making the successive dilutions. Foaming in dilute solutions would, of course, affect the concentration of the soap in the underlying liquid.

Organisms.—The *B. typhosus* used was the Rawlings strain obtained from the Army Medical School. The pneumococcus was a stock culture of type 1. The *Streptococcus haemolyticus* was originally obtained from a throat culture and the *Staphylococcus aureus* had been recently isolated from cystitis due to mixed *B. coli* and *staphylococcus* infection.

Mediums.—The typhoid bacillus and *Staphylococcus aureus* were grown in beef extract broth, P_H 7.4. Twenty-four hour cultures, just previous to use, were filtered through one layer of sterile filter paper. For the pneumococcus and streptococcus, a medium was desired showing the same degree of profuseness in growth as the cultures of typhoid and staphylococcus, and in which growth would easily take place when inoculated with the small number of organisms from the soap solutions. Huntoon's "hormone" broth, P_H 7.6, made according to the formula given by Fisk and Burky,¹² and to which was added 0.25% glucose, was found to be admirably suitable for this purpose. These cultures were used when 18 hours old, as maximum growth had occurred by that time, and no tendency to clumping had appeared. The cultures of pneumococcus and streptococcus were not filtered. Transfers from the soap solutions were made into the same kind of medium in which the organisms had been grown.

EXPERIMENTAL RESULTS

N/5 solutions of the sodium and potassium salts of butyric, valeric, and caproic acids did not kill any of the organisms tested in the longest time interval, namely, 15 minutes. It is hence apparent that the salts of the lower acids are lacking in germicidal properties, as well as in the other characteristics of soaps. Fischer¹⁰ found that the first acid of the series-forming salts resembling soaps was caproic, although

¹² Fisk and Burky, *ibid.*, 1922, 30, p. 128.

caprylic acid was the first one showing these characteristics to any marked degree. The results obtained with the other salts are shown in table 1.

Broth cultures of the 4 organisms do not differ greatly in their resistance to phenol, that is, a 1% solution killed the first 3 in 15 minutes, while a 0.5% solution failed to do so. The staphylococcus was more resistant, requiring a 2% solution to kill in the same length of time. The soaps, on the other hand, vary markedly among themselves, and also in regard to their action on the different organisms.

TABLE 1
KILLING STRENGTH OF SOAPS FOR THE DIFFERENT ORGANISMS
20 C.

	Pneumococcus		Streptococcus		B. typhosus		Staphylococcus aureus 15 Min.
	2½ Min.	15 Min.	2½ Min.	15 Min.	2½ Min.	15 Min.	
Na caprylate.....	N/10	N/20	N/10	N/20	N/5	N/10	—†
K caprylate.....	N/10	N/20	N/10	N/20	N/5	N/10	—
Na caprate.....	N/640	N/640	N/80	N/80	N/5	N/10	—
K caprate.....	N/640	N/640	N/80	N/160	N/5	N/10	—
Na laurate.....	N/5,120	N/10,240	N/640	N/640	N/10	N/20	—
K laurate.....	N/5,120	N/5,120	N/640	N/640	N/20	N/20	—
Na myristate.....	*	N/80	*	*	*	*	*
K myristate.....	N/40	N/40	N/20	N/40	N/10	N/40	—
Na palmitate.....	*	*	*	*	*	*	*
K palmitate.....	N/40	N/80	N/20	N/40	N/40	N/40	—
Na stearate.....	*	*	*	*	*	*	*
K stearate.....	N/40	N/80	N/80	N/80	*	N/20	—
Na oleate.....	N/2,560	N/5,120	N/160	N/320	—†	—†	—
K oleate.....	N/2,560	N/5,120	N/80	N/160	—	—	—
Na linoleate.....	N/2,560	N/5,120	N/320	N/640	—	—	—
K linoleate.....	N/2,560	N/5,120	N/160	N/320	—	—	—
Na linolenate.....	N/5,120	N/10,240	N/1,280	N/1,280	—	—	—
Phenol.....	1%	1%	1%	1%	2%	1%	2%

† The minus sign indicates that the strongest solution tested did not cause the death of the bacteria.

* Sodium myristate, palmitate, and stearate formed gels that were too firm at 20 degrees to be tested in a dilution stronger than N/80. Potassium stearate could not be tested in a dilution stronger than N/20.

The outstanding feature of table 1 is the resistance of the staphylococcus to all the soaps. Two strains of *Staphylococcus albus* were also tested and showed the same resistance, while a third strain of *Staphylococcus albus*, isolated from a recurring furunculosis, differed markedly from the foregoing cultures in that it was killed in 15 minutes as follows: sodium linolenate N/80, sodium and potassium laurate, N/40; other soaps, either N/10 or N/20. Until the reason for this difference in resistance of strains of staphylococcus is understood, or can be associated with other characteristics, it is, of course, conservative to consider the staphylococcus completely resistant.

The bactericidal titers of the potassium and sodium salts of the same acid are the same, or else lie close together, indicating that varying the fatty acid radical is of far more importance in affecting the germicidal power of a soap than varying the alkali. The sodium soaps of myristic, palmitic and stearic acids, as pointed out by Fischer, possess very high gelation capacities, and hence could not be tested in the same strength as the corresponding potassium salts.

The caprylates (table 1) possess rather limited germicidal power; the caprates are more effective, while with the laurates there is a sudden increase in germicidal power, especially for the pneumococcus and streptococcus. A N/10,240 solution (0.002%, that is, approximately 1:50,000) of sodium laurate kills the pneumococcus in 15 minutes, while a N/640 solution (0.035%) kills the streptococcus in the same length of time. Averaging the killing strength for the pneumococcus for 2½ and 15 minutes would give sodium laurate a "pneumococcus phenol coefficient" of something over 200.

The activity of the myristates, palmitates, and the stearates toward the pneumococcus and streptococcus shows a decided drop from the laurates. The 15 minute killing strength of their potassium salts for these organisms is in the neighborhood of N/40 (about 0.7%). This is also their approximate killing strength for the typhoid bacillus; hence they, in contrast to the laurates, show no selective action for the pneumococcus and streptococcus.

The salts of the unsaturated acids are very germicidal toward the pneumococcus and streptococcus, while they seem to be absolutely inert toward *B. typhosus*. The findings of both Reichenbach and Lamar are thus shown to have been exact.

Comparing the stearate, oleate, linolate, and linolenate, their activity in a general way increases with the degree of chemical unsaturation, as noted by Lamar. Lamar, however, did not consider unsaturation the sole factor concerned. Unsaturation, of course, has no bearing on the very high bactericidal titer of sodium laurate. Nor can the activity of the different soaps be correlated with their "solubility," for if this were the case, one would expect the potassium salts to be very much more active than the corresponding sodium salt. However, if the sodium soaps alone are considered, only those possessing a high degree of "solubility" show marked germicidal activity toward the pneumococcus and streptococcus.

Regarding the action of the soaps on *B. typhosus*, it may be pointed out that potassium palmitate, being the only one that kills in $2\frac{1}{2}$ minutes in a N/40 solution, is the most efficient. This again agrees with the conclusions of Reichenbach.² The corresponding sodium salt, however, gels too easily to be tested in this concentration. Potassium soaps, with the exception of the official *Sapo mollis*, have limited application, and this, being prepared from linseed oil, contains little, if any, potassium palmitate.

The germicidal titer of the various soaps for *B. typhosus* (N/10 to N/80, that is, 1.7 to 0.4% for 15 minutes) seems limited when it is compared with the very high figures for the pneumococcus and streptococcus. Yet, from the standpoint of hygiene, it is believed to be high enough to aid in the prevention of the spread of disease, although it

TABLE 2
INFLUENCE OF THE PRESENCE OF 50% SERUM ON THE ACTION OF SODIUM LAURATE AS TESTED
WITH *B. TYPHOSUS* AND *PNEUMOCOCCUS* I.
20 degrees, 15 minutes

Concentration of Soap	<i>B. typhosus</i>		<i>Pneumococcus</i>	
	Control	50% Serum	Control	50% Serum
N/10.....	—	—	—	—
N/20.....	—	—	—	—
N/40.....	+	+	—	—
N/80.....	+	+	—	—
N/160.....	+	+	—	+
N/320.....	+	+	—	+

— signifies no growth; + signifies growth.

would perhaps be premature to attempt to apply it practically from this purely theoretical study alone.

Among the soaps studied, sodium laurate seems to possess the most general germicidal properties. Its activity toward the pneumococcus and streptococcus is of a very high degree, while it is definitely germicidal for *B. typhosus*. The resistance of *Staphylococcus aureus*, however, precludes the use of soaps as general antiseptics.

INHIBITORY EFFECT OF SERUM AND BROTH

Lamar³ showed that the presence of serum exerts an inhibitory effect on the bactericidal activity of soap exactly as the presence of serum prevents the hemolytic action of soap. It was considered desirable to ascertain something about the amount of serum necessary to bring about this inhibition of action. Table 2 shows the effect of the presence

of 50% serum on the action of sodium laurate as tested with the pneumococcus and *B. typhosus*. Its action on *B. typhosus* is apparently uninfluenced, while the killing strength in 15 minutes for the pneumococcus is lowered from N/10,240 (table 1) to N/80. The inhibitory effect of the action on the *Streptococcus* was even more marked, the presence of 5% serum lowering the 15 minute killing strength of sodium laurate from N/640 to N/80; of sodium oleate, from N/160 to N/20; and of sodium linoleate, from N/640 to N/80.

It was also found that diluting sodium laurate in broth instead of in distilled water lowered its 15 minute killing strength for the pneumococcus to N/160.

TABLE 3
EFFECT OF TEMPERATURE ON SOAPS AS TESTED WITH *B. TYPHOSUS* FOR 15 MINUTES

	Concentration of Soap		
	N/20	N/40	N/80
Na laurate 4 C.	+	+	+
Na laurate 37 C.	—	—	+
K palmitate 4 C.	+	+	+
K palmitate 37 C.	—	—	+
Na oleate 4 C.	+	+	+
Na oleate 37 C.	—	+	+

TABLE 4
SODIUM LAURATE, N/640. EFFECT OF TEMPERATURE AS TESTED WITH PNEUMOCOCCUS I.
TRANSFERS MADE AT DIFFERENT TIME INTERVALS

Temperature	Exposure in Minutes					
	1	2½	5	10	20	30
4 C.	+	+	+	+	+	+
20 C.	+	—	—	—	—	—
37 C.	—	—	—	—	—	—

EFFECT OF TEMPERATURE

Jolles¹³ claimed to have observed that soaps were more active as germicides at low temperatures. Rasp¹⁴ was unable to confirm this, although the work of Jolles is apparently accepted by Rideal and Rideal¹⁵ in their very comprehensive work on chemical disinfectants. This point is of so much practical and theoretical importance that the work of Rasp was repeated. Table 3 shows the results with *B. typhosus* and table 4 those obtained for the pneumococcus with a constant concen-

¹³ Ztschr. f. Hyg. u. Infektionskr., 1895, 19, p. 130.

¹⁴ Ibid., 1907-08, 58, p. 45.

¹⁵ Chemical Disinfection and Sterilization, 1921.

tration of sodium laurate, in which the time of exposure was varied. There seems to be no question but that raising the temperature increases markedly the germicidal activity of the soaps, even sodium oleate, which was inert at 20 degrees in a N/5 solution, becoming distinctly germicidal toward *B. typhosus* at 37 degrees.

DISCUSSION

Behrend¹⁶ believed that the germicidal action of soap was to be attributed solely to the alkali present. This opinion is no longer held, although it is of interest in this connection to note that the detergent action of soap was formerly attributed chiefly to the hydrolyzed alkali. The true explanation of the detergent action of soaps rests on their colloidal nature, and the literature of this subject is reviewed by McBain.¹⁷ McBain has also shown that the degree of hydrolysis in soap solutions is much less than is generally supposed, being about 0.001N $\overline{\text{OH}}$ for most soap solutions. Alkali of this concentration has no effect on the organisms used in this work. The pneumococcus was found, however, to be relatively susceptible to the action of alkali, being killed in 15 minutes by a N/20 solution of NaOH. Nichols⁶ took the hydrogen-ion concentration, using colorimetric titration, of the soap solutions with which he worked, and found that alkali solutions of the same hydrogen-ion concentration as these solutions had no effect on the pneumococcus or streptococcus.

Reichenbach² believed that the germicidal action of soap was to be explained by the mutual summation of the effects of the hydrolyzed alkali and the unchanged soap.

The results given in table 1 show that no comprehensive theory of the action of soaps on bacteria can be given without taking into consideration differences in the various bacteria. Why should the pneumococcus be killed by a N/10,240 solution of sodium linolenate, while the typhoid bacillus is apparently unaffected by the same substance in a concentration 2,000 times as strong? Our state of knowledge at present does not permit the giving of a satisfactory answer to this question. The action of some of the dyes on different organisms has been correlated with that of the Gram stain, but there seems to be no such relationship in the case of the soaps.

¹⁶ Ztschr. f. Hyg. u. Infektionskr., 1890, 9, p. 414.

¹⁷ Third Report on Colloid Chemistry, Brit. Assn. for Adv. of Science, 1920.

Berczeller¹⁸ has shown that the germicidal activity of various substances investigated by him varied directly, with a few exceptions, with their depressant action on surface tension. Surface tension depressants are absorbed at the bacterium-medium interface, and would have more effect than germicides without this property. A discussion of this point is given by Rideal.¹⁹ Soaps have a marked effect in lowering surface tension, and this effect continues well into dilute solutions. It is probable that the action of soaps on bacteria is enhanced by this effect. Also, the action of soaps on the pneumococcus and streptococcus is to some extent inhibited by the presence of serum. Du Noüy²⁰ has observed that the depressing effect of sodium oleate on surface tension is markedly lessened by the presence of blood serum, that is, serum exerts a sort of "buffering" action so far as changes in surface tension are concerned. This observation of Du Noüy's and the inhibition of the germicidal effect of soaps by serum are possibly related, although part of the inhibition of the germicidal effect may be due to the precipitation of soap by the calcium and magnesium salts present in blood serum.

Even if there is a relationship between the bactericidal activity of soaps and their lowering of surface tension, it still does not explain the selective action of the various soaps, nor the relative resistance of the typhoid bacillus and the marked resistance of the staphylococcus. It may be noted, however, that there is no method available for directly measuring the interfacial surface tension between the liquid and bacterium, and it is usually assumed that the tension here varies with that of the liquid-air interface. However, as pointed out by Rideal,¹⁹ this is by no means universally true. This affords ground for suspecting that the interfacial tension at the bacterium-soap solution interface may not be the same for all bacteria, and hence there is a possibility that the germicidal properties of soaps and the resistance of bacteria may be more closely correlated with surface tension in the future.

CONCLUSIONS

Soaps prepared from pure fatty acids differ markedly in their germicidal properties, although sodium and potassium soaps of the same fatty acid do not differ greatly in this respect.

The lowermost members of the fatty acid series possess no or limited germicidal properties against the organisms tested.

¹⁸ *Biochem. Ztschr.*, 1914, 66, p. 202.

¹⁹ Fifth Report on Colloid Chemistry, Brit. Assn. for Adv. of Sc., 1923.

²⁰ *Jour. Exper. Med.*, 1922, 36, p. 115.

Staphylococcus aureus possesses marked resistance to the action of soaps, not being killed by any of them under the conditions of these experiments.

The pneumococcus shows marked susceptibility to the action of the laurates, oleates, linoleates, and linolenate, being killed in 15 minutes by a N/10,240 (approximately 1:50,000) solution of sodium laurate. A 1% solution of phenol is required to kill under the same conditions.

The streptococcus is killed by the same soaps as the pneumococcus, although it does not possess the same extreme susceptibility.

Bacillus typhosus is considerably more resistant to the action of soaps, although killed in 15 minutes by N/20 to N/40 (1.5 to 0.5%) solutions of the soaps of the saturated acids, from lauric to stearic inclusive. A 1% solution of phenol is also required to kill under similar conditions. On the other hand, *B. typhosus* is markedly resistant to the soaps of the unsaturated acids, despite the strong action of the latter on the pneumococcus and streptococcus. These soaps hence show marked selective activity for the pneumococcus and streptococcus.

The soaps of myristic, palmitic, and stearic acids kill the pneumococcus, streptococcus, and typhoid bacillus in about the same concentration, and hence exhibit no marked selective action.

The laurates, in that they are strongly germicidal toward the pneumococcus and streptococcus, and at the same time show definite germicidal properties toward the typhoid bacillus, seem to possess the most general action as germicides. The marked resistance of *Staphylococcus aureus*, however, of course, limits the use of soaps as general antiseptics.

The presence of blood serum and broth has an inhibitory effect on the action of the soaps, although the presence of even 50% serum did not completely destroy the action of the stronger solutions.

In common with other germicides, soaps act best at higher temperatures.

No attempt is made to explain the germicidal effect of soap solutions, although it may possibly be related to their depressor effect on surface tension.

PERMEABILITY OF THE RABBIT PLACENTA TO PRECIPITINS AND TO TYPHOID AGGLUTININS

M. F. GUYER AND E. A. SMITH

From the Zoological Laboratory, University of Wisconsin, Madison

In connection with various experiments concerned with the induction of defects in unborn young, it became imperative to determine whether or not, in rabbits, antibodies pass from the mother to the fetus through the placenta. Examination of the literature led only to confusion, since one found such placental transmission in mammals both positively affirmed and vigorously denied. As we have pointed out,¹ there are various factors that may be responsible for such differences in result, one of the most probable being the fact that the placental relationships of the young to the mother differ much in the different kinds of mammals. Thus, the pig has a diffuse type of placentation in which the chorionic villi are loosely associated with the uterine wall. The carnivora have a somewhat more intimate girdle-like or zonary type. Sheep, cows, and goats have a cotyledonary type in which the chorionic outgrowth comes in contact with the uterine mucosa in a number of separate places. In the rabbit and the guinea-pig, on the other hand, with discoidal placenta, the connection between fetus and mother is, as in man, much more intimate. It is a well established fact, moreover, that young mammals² may acquire antibodies from the milk of an immunized mother or foster mother; hence, to test the matter of placental transmission, all possibility of the young animal having suckled must be excluded.

Since we were concerned primarily with rabbits, we have confined our experiments to them, and whatever the situation may be with reference to other mammals, we are sure, as mentioned before,¹ that both precipitins and agglutinins pass to the fetus through the placenta. Moreover, in order to have more definite knowledge of the exact relation of the blood capillaries in the fetal placenta to the maternal blood at different periods of gestation, we have had an extensive reexamination made of the rabbit's placenta histologically and by means of double injections, by Harland W. Mossman (in press). From the

Received for publication, Aug. 1, 1924.

¹ Jour. Infect. Dis., 1923, 33, p. 22.

² Ibid., 1923, 33, pp. 20-22.

twelfth day, notwithstanding the so-called plasmodial covering, there are thin places where little or nothing but the fetal endothelium separates the maternal from the fetal blood, and from the twenty-second day on the membrane separating the two streams seems to consist mainly of only a single layer of fetal endothelium. With the blood capillaries of the fetal part of the placenta thus bathed directly in the maternal blood, there would seem to be favorable conditions for interchanges in the two blood streams. Even apart from diffusion, it is difficult to see why, with the violent kicking and jerking about that rabbits do when handled, actual rupture of the delicate endothelium should not occur occasionally and thus permit direct admixture of the maternal with the fetal blood.

DOES ANTIGEN PASS THROUGH THE PLACENTA?

In order to determine whether antigen passes through the placenta, six rabbits received intravenous injections with sheep serum. Two aborted their young, leaving only four of the fetal young on which satisfactory precipitin tests could be made. The blood of these fetuses was titrated with the serum of rabbits that had previously been immunized against the sheep serum. In every case, the test on the maternal blood was positive; on the fetal, with one doubtful exception, negative. The individual records follow:

ANIMAL 1.—Female 645.1 was mated, May 27, 1922. Later she was injected with normal sheep serum as follows: June 19, 5 c.c.; June 21, 7 c.c.; June 23, 7 c.c.; June 24, 10 c.c. On June 24, about an hour after the injection, she was killed and bled. The 7 uterine young were carefully removed and bled by slitting the thorax and cutting the jugular veins. The blood was allowed to drip into centrifugal tubes.

Titration of maternal and of fetal blood were made in all dilutions from 1:80 to 1:10,240 against the serum of 2 rabbits which had been immunized to sheep blood. Each of the latter, 98B3 and 111A1, showed titers of 1:2,560 according to the ring test and of 1:5,120 by the flocculation test. The fetal serum remained negative in all dilutions.

ANIMAL 2.—Female P1 was mated, June 28, 1922. She was injected with normal sheep serum as follows: July 21, 5 c.c.; July 22, 5 c.c.; July 25, 5 c.c.; July 26, 10 c.c. She was killed and bled one hour after the injection, July 26. The 5 fetuses were treated as in case 1. The antiserum against which the maternal and the fetal blood were titrated had a titer of 1:10,240 against sheep serum. The maternal blood gave positive precipitin reactions in dilutions of 1:10,240, with this antiserum, by both the ring and flocculation test. The fetal serum remained negative in all dilutions.

ANIMAL 3.—Female P2 was mated, July 5, 1922. She was injected with 5 c.c. of normal sheep serum on July 29 and on August 1, and with 9 c.c. on August 2. She was killed and bled an hour later. The 8 fetuses were

treated as in the previous experiments. The rabbit antiserum against which the respective bloods were titrated showed a titer of 1:10,240 against sheep serum. The maternal blood gave positive precipitin reactions in all dilutions up to and including 1:10,240 by both ring and flocculation tests. The fetal blood gave doubtful feeble positive reactions in dilutions 1:40, 1:80, 1:160, and 1:320 by the ring test, and negative reactions in all dilutions by the flocculation test.

ANIMAL 4.—Female PE was mated, Sept. 21, 1922. She was injected with 8 c.c. of normal sheep serum on Oct. 18, and with 10 c.c. on Oct. 20. She was killed and bled on Oct. 20, one hour after the injection. She contained only one fetus. The rabbit antiserum used for titration had a titer of 1:10,240 against sheep serum. The maternal blood serum gave positive precipitin reactions in dilutions up to 1:640; the fetal serum remained negative in all dilutions.

PENETRATION OF THE PLACENTA BY PRECIPITINS

In the experiments to determine whether or not precipitins pass from the mother to the fetus in rabbits, either goat serum or sheep serum was injected intravenously into supposedly pregnant females at such a time as to make the curve of antibody formation reach its peak shortly before the date of parturition. The females were killed at this time, and the uterine young were carefully removed so as to avoid all chance of contamination by the blood of the mothers. In some instances, the amniotic fluid as well as the fetal blood was tested.

Thirteen female rabbits in all were used in this series of tests, but as four aborted or proved to be infertile, the conclusion is based on comparison of the blood of nine mothers with the blood of their fetal young.

The maternal blood to be tested was drawn from the median artery of the ear while the mother was still alive. She was then killed in a gas chamber. The young, including the fetal part of the placenta, were carefully removed with membranes intact, and washed in distilled water or in sterile normal salt solution. The amnion was then ruptured and the amniotic fluid discarded or set aside for testing. The fetuses were again washed in distilled water. The fetal blood was obtained either directly from the end of the umbilical cord or more frequently by slitting the thoracic region and draining the blood from the heart and jugular vein into centrifugal tubes.

A series of dilutions of goat serum or of sheep serum, depending on which had been originally used as antigen, were made in precipitin tubes in each case for the maternal serum, the fetal serum, and the amniotic fluid, respectively. The drop (0.1 c.c.) of antiserum to be tested was always introduced at the bottom of each tube, since this method gives a smooth layering of the two reagents employed. The ring test was

read after 20 minutes; the contents were shaken thoroughly, and the resulting "flocculation test" was then read and recorded at the end of 2 hours. Control tubes containing, respectively, 0.1 c.c. antigen and 0.1 c.c. maternal antiserum (or 0.1 c.c. fetal antiserum) each in 0.5 c.c. normal salt solution, were always included in each series of dilutions.

In every case, in both mother and fetuses, the results of such precipitin tests were positive. Usually the titer of the fetal serum ran slightly below that of the mother. This is due probably to the fact that the fetal serum was diluted somewhat with the water used in washing the fetuses. Specific gravity tests of the serum in question bear out this interpretation.

The titers of the amniotic fluid invariably ran lower than those of the fetal serum.

Since the results in all cases were similar, it would be needless repetition to give the data for each female and her fetuses in detail. The following representative case will serve for exemplification:

Female F2 was bred, Jan. 16, 1922. She received intravenous injections of 5 c.c. of goat serum on February 2; 6 c.c., on February 4, and 9 c.c. (6 c.c. in the forenoon and 3 c.c. in the afternoon) on February 6. On February 13, she was bled from the median artery of each ear, and 26.5 c.c. of blood was thus obtained. She was then killed and the young, 3 alive and 6 dead (the latter partly resorbed), were removed. The live young were thoroughly washed in distilled water, the amnion ruptured, and the fetuses again washed in distilled water. The fetal blood was obtained by slitting the thorax and cutting into the heart and jugular veins. The blood was allowed to drain into centrifugal tubes, and when clotted it was centrifuged and the serum drawn off. The serum of the maternal blood was also separated out by centrifuging.

Titration of maternal and of fetal blood were made in all dilutions of goat serum ranging from 1:10 to 1:10,240. The maternal serum gave positive precipitin reactions in dilutions up to 1:5,120 by both the ring and the flocculation test. The fetal serum showed positive results in dilutions up to 1:1,280 by the ring test and up to 1:2,560 by the flocculation test. All controls remained negative.

From the foregoing series of experiments, since antigen rarely, if ever, passes through the placenta, it is evident that the antibodies (precipitins) found in the fetuses must have reached the fetal blood directly from the maternal blood and were not formed by the fetuses through a reaction of antigen in their own circulation.

NON-PENETRATION OF COLLODION MEMBRANE BY PRECIPITINS

To determine whether precipitins dialyze readily through an ordinary collodion membrane, a dialyzer consisting of a thistle tube with a collodion membrane over its mouth was used. Normal serum diluted 1:3 was put into a sterile container; then the mouth of the membrane-

covered thistle tube containing the antiserum to be tested, likewise diluted 1:3, was immersed in it and set in the icebox for from 24 hours to several days in the different experiments.

In a parallel series, normal salt solution instead of normal serum was used in the outer vessel. Also all tests were made in duplicate.

Antiserum from 4 rabbits was tested at intervals from November, 1922, to February, 1923. Precipitin formation was induced in the ordinary way by 4 successive injections (usually 5, 5, 7 and 10 c.c., respectively) of normal sheep serum.

Titration of the serum of male 1 for precipitins gave a positive reaction in dilutions of 1:2,560 by the ring test and of 1:5,120 by the flocculation test; of female 2, 1:10,240 by both ring and flocculation test; of female 4, 1:320 by the ring and 1:1,280 by the flocculation test; and female 5, 1:1,280 by the ring and 1:2,560 by the flocculation test; rabbit number 3 died. In not a single case, however, did the precipitin pass through the collodion membrane, for all tests of the surrounding diffusate, whether into normal serum or salt solution, remained negative. We may conclude, therefore, that precipitin developed in rabbit serum against sheep serum as antigen does not pass by simple dialysis through pores the size of those in an ordinary collodion membrane.

PENETRATION OF THE PLACENTA BY AGGLUTININS

To determine whether or not agglutinins pass through the placenta in rabbits, females were immunized to typhoid bacilli, bred, and then killed shortly before the young were to be born. Precautions against contamination with the mother's blood or otherwise were taken as in the experiments with precipitins.

In general, our standard method of typhoid immunization was as follows: intravenous injections of 0.5 c.c. of vaccine the first day, 1.0 c.c. the third day, 1.5 c.c. the fifth day, $\frac{1}{4}$ agar slant the eighth day, and $\frac{3}{4}$ agar slant the tenth day. A $\frac{1}{4}$ agar slant consists of 0.5 c.c. of a suspension of a 16-hour culture of *B. typhosus* washed with 2.0 c.c. of 0.9% salt solution; a $\frac{3}{4}$ agar slant consists of 1.5 c.c. of such a suspension.

The so-called "macroscopic" agglutination test was used. The antigen consisted of a typhoid suspension prepared by using 10 c.c. of a normal salt solution on a 24-hour agar slant culture. The usual antigen and antiserum controls were employed.

Of the 15 pregnant rabbits used in the experiments, 6 died or aborted their young so that the tests were made on 9 mothers and their fetal young.

In every case, the fetal serum gave positive reactions in dilutions similar to or slightly lower than that of the mother. The amniotic fluid in those cases in which it was tested likewise gave positive agglutination reactions, but in dilutions lower than did the embryonic blood. The lower titer of the fetal blood is probably due, as in the experiments with precipitins, to slight dilutions of the fetal serum with the water in which the fetuses were washed.

Because of the general similarity of the result in the 9 cases, only the 2 following are mentioned specifically by way of exemplification:

Female 53A2 was immunized to typhoid in the usual way, bred Feb. 27, 1922, and killed March 24. The blood was collected from the 8 fetuses, after washing them, by slitting the ventral thoracic wall and draining directly from the heart and the jugular veins. The blood serum of the mother produced agglutination in suspensions of typhoid bacilli in dilutions as high as 1:2,560; that of the fetuses, in dilutions up to 1:640.

Female 90A6 was immunized to typhoid in the usual way, bred April 6, 1922, and killed, May 5. The 6 fetuses were bled as in the preceding experiment. The blood serum of the mother produced agglutination in dilutions as high as 1:2,560; that of the fetuses in dilutions up to 1:1,280. The amniotic fluid, on the other hand, produced agglutination in dilutions only as high as 1:320.

SUMMARY

The blood of fetal rabbits shows positive precipitin reactions toward proteins that have been injected into the mother. Likewise, the blood of fetal young of mothers that have been immunized to typhoid bacilli agglutinate typhoid bacilli. Precipitins do not dialyze through an ordinary collodion membrane nor does such antigen as normal sheep serum (with doubtful exceptions) pass through the normal rabbit placenta.

HOW SALT PRESERVES

GEORGE E. ROCKWELL AND EDWIN G. EBERTZ

From the Department of Bacteriology and Hygiene of the University of Cincinnati

Various workers have concluded that sodium chloride prevents bacterial growth merely through its ability to dehydrate protein.¹ Can this be true, or are several factors involved?

If sodium chloride inhibits bacterial growth by means of dehydration, then other equally efficient dehydrating salts should be as capable of preventing bacterial growth. It has been shown that magnesium sulphate has a greater dehydrating effect on proteins than sodium chloride.² It is shown in table 1 that it is not as efficient in preventing bacterial growth.

TABLE 1
THE LIMITING CONCENTRATION OF SALTS ON THE GROWTH OF STAPHYLOCOCCUS AUREUS

	N	1.5	2	2.5	3	4	5
NaCl	+	+	+	+	+	+	+
MgSO ₄ .7H ₂ O	+	+	+	+	+	+	+

In the experiment illustrated in table 1 and in all of the following experiments, the medium contained 3 gm. of meat extract, 10 gm. of peptone, and 5 gm. of sodium chloride per liter. The extract contained per 100 gm., 7.78 gm. nitrogen, 8.5 gm. sodium chloride and 22.85 gm. water. The peptone contained per 100 gm., 14.72 gm. nitrogen, 0.915 gm. sodium chloride and 7.83 gm. of water. This gave a broth of a P_H 6.6. The various salt concentrations of broth were obtained by taking 1 volume of standard broth, to which was added the desired amount of salt, and then this salt broth mixture was diluted to 2 volumes with distilled water. The salt broth mixtures were inoculated with one loopful of a suspension of *Staphylococcus aureus* in salt solution.

Table 1 shows that magnesium sulphate, although a better dehydrator of protein than sodium chloride, is not as efficient in inhibiting bacterial growth. Therefore we must conclude that the preserving effect of sodium chloride involves more than its dehydrating capacity.

Received for publication, Aug. 1, 1924.

¹ Lafar: *Technical Mycology*, 1910; Kendall: *Civilization and the Microbe*, 1923. Taylor, H. F.: *Principles Involved in the Preservation of Fish by Salt*, 1920.

² Fischer, M. H.: *Oedema and Nephritis*, 1921.

Does the chlorine ion of sodium chloride have a direct effect on bacteria? That it does is illustrated by comparing the inhibitive action on bacterial growth of various salts (table 2).

TABLE 2
SHOWING THE LIMITING CONCENTRATIONS OF VARIOUS SALTS ON THE GROWTH OF
STAPHYLOCOCCUS AUREUS

Normality	1	1.5	2	2.5	3	4
NaCl	+	+	+	—	—	—
NaBr	+	+	+	+	+	+
Na ₂ SO ₄	+	+	+	+	+	+
MgSO ₄	+	+	+	+	+	+
MgCl ₂	+	+	+	—	—	—
Sodium citrate	—	—	—	—	—	—
Mg. citrate	—	—	—	—	—	—

Table 2 shows that the limiting concentration of the various sodium salts is different, and we must conclude that the order of toxicity is citrate, chloride, bromine, sulphate; further that the magnesium radicle is not very toxic, in fact less toxic than sodium, although it is a more efficient dehydrator than sodium; also that magnesium chloride and sodium chloride inhibit the growth at about the same normality, e. g., it is the chloride radicle which dominates.

Sodium chloride solution is a poor solvent for oxygen, hence it tends to prevent growth by oxygen removal, as illustrated in table 3.

TABLE 3
THE EFFECT OF OXYGEN AND CARBON DIOXIDE ON THE GROWTH OF STAPHYLOCOCCUS AUREUS
IN SALT BROTH

% NaCl	2	4	6	8	10	12	14	16
Control	+	+	+	+	±	—	—	—
O ₂ passed through.....	+	+	+	+	+	+	+	±
CO ₂ passed through.....	±	—	—	—	—	—	—	—

Table 3 reveals that the passing of oxygen through a brine causes an increased growth, while the passing of carbon dioxide through the brine decreases the growth. From this one can conclude that sodium chloride interferes with bacterial growth, partly through its ability of excluding oxygen, and sensitizing the organisms to carbon dioxide.

High concentrations of salt interfere with enzyme action. It is shown in table 4 that one of the ways in which salt prevents decomposition of proteins is through its interference with the action of proteolytic enzymes.

Table 4 shows that rapid liquefaction is checked before the growth of proteus is prevented.

TABLE 4
GROWTH OF PROTEUS ON COAGULATED BLOOD SERUM, CONTAINING SODIUM CHLORIDE
INCUBATED AT 24 C. FOR 3 DAYS

% NaCl	1	2	3	4	5	6	7	8	9	10	11
Growth	+	+	+	+	+	+	+	+	+	±	—
Liquefaction of blood serum.....	+	+	±	—	—	—	—	—	—	—	—

We have shown in previous articles that the inhibitive action of sodium chloride on bacterial growth is affected by the amount of protein present, the temperature and the reaction;³ hence it can be readily understood how variations in these conditions in turn affect the various ways in which salt preserves.

SUMMARY

The preserving of proteins with salt involves more than its dehydration effect, there being at least four factors, namely, dehydration, direct effect of chlorine ion, removal of oxygen, sensitization against carbon dioxide and interference with rapid action of proteolytic enzymes.

³ Jour. Am. Leather Chemists Assn., 1923, 18, p. 233; 1924, 19, p. 369.

THE EFFECT OF CL. SPOROGENES ON CL. BOTULINUM *

EDWIN O. JORDAN and GAIL M. DACK

From the Department of Hygiene and Bacteriology of the University of Chicago

Cl. botulinum is, at least in some parts of the world, a relatively common organism.¹ Botulism, however, is relatively uncommon. The facts need interpretation, since in the United States where the practice of canning both in the home and in large factories is widespread and the spores *Cl. botulinum* are widely distributed, botulism has been among the rarest of human diseases.

It is significant also that in numerous instances in which the presence of botulinus toxin has been demonstrated in certain jars or cans of food, other containers of the same food preserved at the same time and to all appearances in the same manner have proved entirely innocuous. Usually, only a small proportion of the cans of an incriminated shipment contain botulinus toxin. Thus Koser, Edmondson and Giltner,² who examined 174 cans of spinach obtained either from various shipments believed to be connected with botulism outbreaks or from lots suspected of being imperfectly processed, found *Cl. botulinum* or its toxin in but 6. The same observers in making experimental inoculations of cans of spinach with *Cl. botulinum*, although using for inoculum approximately 800,000 spores, found the development of the organism and its toxin production somewhat irregular. Ripe olives, which have been implicated in a number of botulism outbreaks, have proved quite unsuitable for the experimental production of toxin. Schoenholz, Esty and Meyer,³ who made an exhaustive study of the effect of the inoculation of *Cl. botulinum* into various canned foods, state that "It must be emphasized that growth and toxin production, irrespective of the suitable reaction, are very irregular in sound, unbroken, ripe olives. Potent toxins have not been demonstrated in any of the experimentally inoculated containers."

Received for publication, Oct. 21, 1924.

* This study was aided by a grant from the National Cannery Association.

¹ Meyer, K. F., and Dubovsky, Bertha J.: *Jour. Infect. Dis.*, 1922, pp. 31, 541, 559, 595, and 600. Coleman, G. E.: *Ibid.*, p. 556. Schoenholz, P., and Meyer, K. F.: *Ibid.*, p. 610. Hall, I. C., and Peterson, Emelia C.: *Jour. Bacteriol.*, 1924, 9, p. 20.

² *J. A. M. A.*, 1919, 77, p. 1250.

³ *Jour. Infect. Dis.*, 1923, 33, p. 289.

Evidently the concurrence of those factors necessary for the production of botulinus toxin in canned foods is not common. Among the factors that may conceivably influence the development of *Cl. botulinum* and its toxin production is the presence of other bacteria, and the experiments reported in this paper were carried out to test this possibility. Hall and Peterson⁴ found that certain acid-producing aerobes inhibited toxin production or destroyed botulinus toxin in dextrose broth, a fact in accord with the general experience that it is difficult to demonstrate toxin production by *Cl. botulinum* in soil, feces, etc., unless heated cultures are used.

Cl. sporogenes, whose association with *Cl. botulinum* we have studied, is a rather large rod, forming subterminal spores which swell the rod into the clostridium form. It is actively proteolytic, blackens brain medium and liquefies gelatin. Lactose, saccharose and salicin are not fermented; glycerol is fermented. It is widely distributed in nature and is a common contaminant of anaerobic laboratory cultures.⁵

Strains of *Cl. sporogenes* isolated from soil were compared with laboratory cultures from various sources. Agglutinins were obtained by rabbit inoculation (intravenous, 6 injections) and the identity of the soil strains with the verified laboratory strains was determined culturally and serologically. These strains were used in the experiments that follow.

In table 1 is shown the result of simultaneous inoculation into beef heart medium of a strain of *Cl. botulinum* with one of *Cl. sporogenes*. When only 5 cells of either organism were used, they were counted out accurately by the Barber pipet technic. The estimation of an inoculum of 5,000 cells and over was made with a standardized suspension prepared by the aid of the hemacytometer. One series was heated to 80 C. for 1 hour; the other was left unheated; growth was observed in all tubes after 3 days' incubation at 37 C. Toxicity was tested by injecting mice intraperitoneally with 0.25 c.c. of the centrifuged supernatant fluid after 10, 20 and 30 day incubation periods.

At the close of each test, suitable dilutions of the mixed cultures were inoculated into agar shake tubes and the type of colony that developed carefully noted. In each case in which toxin was present,

⁴ Jour. Bacteriol., 1923, 8, p. 319.

⁵ Hall, I. C.: Jour. Infect. Dis., 1922, 30, p. 445.

Cl. botulinum colonies alone were observed, and in all cultures in which no toxin was produced only colonies of the Cl. sporogenes type seemed to be present. It is recognized, however, that identification by colony formation does not possess absolute value.

TABLE 1
RESULTS OF INOCULATING BEEF HEART MEDIUM SIMULTANEOUSLY WITH CL. BOTULINUM (M7A2) AND CL. SPOROGENES (310-1)

Number Spores Cl. botu- linum	Number Spores Cl. sporo- genes	Toxin in					
		Unheated			Heated 80 C. for 1 Hour		
		10 Days	20 Days	30 Days	10 Days	20 Days	30 Days
5	0	+	+	+	+	+	+
5	0	+	+	+	+	+	+
5	5,000	—	—	—	—	—	—
5	5,000	—	—	—	—	—	—
5	5	—	—	—	—	—	—
5	5	—	—	—	—	—	—
5,000	5	+	+	+	—	—	—
5,000	5	+	±	—	—	—	—

Mouse died, +; mouse showed no symptoms, —; mouse showed symptoms but recovered, ±.

TABLE 2
RESULTS OF INOCULATING BEEF HEART MEDIUM SIMULTANEOUSLY WITH CL. BOTULINUM (M7A2) AND CL. SPOROGENES (310-1)

No. Spores Cl. botulinum	No. Spores Cl. sporogenes	Toxin in					
		Unheated			Heated 80 C. for 1 Hour		
		5 Days	10 Days	25 Days	5 Days	10 Days	25 Days
5,000	0	+	+	+	+	+	+
5,000	5,000	+	+	+	+	—	—
5,000	50,000	—	—	—	—	—	—
5,000	500,000	—	—	—	—	—	—
5,000	5,000,000	—	—	—	—	—	—
50,000	0	+	+	+	+	+	+
50,000	5,000	+	+	+	+	+	+
50,000	50,000	+	+	+	+	—	—
50,000	500,000	—	—	—	—	—	—
50,000	5,000,000	—	—	—	—	—	—
500,000	0	+	+	+	+	+	+
500,000	5,000	+	+	+	+	+	+
500,000	50,000	+	+	—	+	+	+
500,000	500,000	+	+	+	—	—	—
500,000	5,000,000	—	—	—	—	—	—
5,000,000	0	+	+	+	+	+	+
5,000,000	5,000	+	+	+	+	+	+
5,000,000	50,000	+	+	+	+	+	+
5,000,000	500,000	+	+	+	+	+	+
5,000,000	5,000,000	—	—	—	+	—	—

A second set of observations using larger numbers of organisms for inoculation was then made. Suspensions were prepared from alkaline egg cultures and washed and suspended 3 times in salt solution. The

cultures used in making the suspensions had been incubated for 13 days at 37 C. and were mostly spores. The suspensions were standardized with the hemacytometer, several counts being made of the various dilutions, and in addition quantitative dilutions were made into deep agar shake tubes and the resultant colonies enumerated.

It is plain from tables 1 and 2 that when *Cl. sporogenes* is considerably in excess in beef heart mediums no botulinus toxin is formed, that when approximately equal numbers of *Cl. botulinum* and *Cl. sporogenes* are inoculated a definite inhibition of the formation of botulinus toxin results, and that a marked excess of *Cl. botulinum* cells (e. g., 10:1) apparently allows botulinus toxin to be formed in the same degree as if no *Cl. sporogenes* were present.

There is some indication that heating at 80 C. for 1 hour usually injures the cells of *Cl. botulinum* or impairs their toxin-producing power to such a degree that in mixtures with *Cl. sporogenes* toxin formation is inhibited or greatly delayed, while in corresponding unheated mixtures toxin production takes place. Whether this difference between heated and unheated mixtures is due to a disproportion of the two organisms in the mixture or to an injury of the spores, was not determined. Dickson and his collaborators have shown that heated spores of *Cl. botulinum* manifest retarded germination.

When toxin production occurs in mixed cultures, there is a tendency for the toxin to disappear on continued incubation, while the corresponding pure cultures hold their toxicity throughout the period of observation. This may be interpreted as indicating a destruction of botulinus toxin by *Cl. sporogenes*.

SUMMARY

Simultaneous inoculation of equal numbers of spores of *Cl. sporogenes* and *Cl. botulinum* into beef heart medium interferes with the development of botulinus toxin.

Cl. sporogenes may either prevent the development of botulinus toxin altogether, may diminish the amount that is produced, or may cause the early disappearance of the toxin.

The inhibiting effect of *Cl. sporogenes* on the production of toxin by *Cl. botulinum* is most marked when *Cl. sporogenes* spores are in the majority or in nearly equal numbers; if botulinum spores are numerous

⁶ J. A. M. A., 1922, 79, p. 1239; Jour. Infect. Dis., 1923, 33, p. 274; see also Esty and Meyer: Jour. Infect. Dis., 1922, 31, p. 650.

in relation to those of *Cl. sporogenes*, toxin production may be hindered slightly or not at all.

Heating the culture medium containing the mixture of *Cl. sporogenes* and *Cl. botulinum* hinders the production of toxin or lessens the amount that is produced.

AN IMPROVED METHOD FOR ANAEROBIC CULTURES

GEORGE E. ROCKWELL

From the Department of Bacteriology and Hygiene, University of Cincinnati

In previous articles,¹ it was shown that the various ways of procuring anaerobic conditions are not alike in their effect on bacterial growth, and the conclusion was that anaerobic life is more than life without oxygen, carbon dioxide being needed in some, if not in all, instances. It was also emphasized that anaerobic conditions as produced by alkaline pyrogallic acid were not satisfactory, because it not only removes the oxygen, but all of the carbon dioxide, even that produced by bacteria when they are present in small numbers. It is apparent that the ideal way of producing anaerobic conditions is by not only removing the oxygen, but also preventing the removal of carbon dioxide and even adding, if possible, small quantities of carbon dioxide. Displacement with gases is too elaborate a procedure for routine laboratory work, hence the following method was devised, which is both simple and satisfactory.

METHOD

Anaerobic conditions are produced by the following modification of Wright's modification of Buchner's method: Pyrogallic acid crystals are placed on the cotton stopper of the culture tube (15-18 mm. x 150 mm.), to which is then added from 1 to 2 c.c. of alkaline water charged with carbon dioxide, and then the tube is quickly sealed with a rubber stopper.

The charged alkaline water was originally made by adding to charged carbon dioxide water, obtained from a soda fountain, 10% of sodium bicarbonate. This solution should be kept sealed and in a cool place. However, the following way has been devised for preparing this solution. A bottle is selected that will hold 500 c.c. of solution. Seventy-five hundredths of a gram of $\text{NaH}_2\text{PO}_4 \cdot 4\text{H}_2\text{O}$ is carefully weighed. This, together with 50 gm. of NaHCO_3 is added to the bottle and accurately diluted to 500 c.c. The bottle is quickly sealed with a rubber stopper, and the solution is set in a cool place.

This solution is so calculated that in each cubic centimeter of solution 0.2 c.c. of carbon dioxide will be liberated at 37 C. and 760 mm. of pressure.

The advantages of this method are: Oxygen is completely absorbed; no carbon dioxide is absorbed as the water is already supersaturated

Received for publication, Oct. 16, 1924.

¹ Jour. Infect. Dis., 1921, 28, p. 352; 1923, 32, p. 98.



Fig. 1. Growth of *B. welchii*, tube 1, aerobic; tube 2, anaerobic as produced by pyrogalllic acid and carbon dioxide charged alkaline water; tube 3, anaerobic by pyrogalllic acid and caustic alkali.

with it, and no alkali capable of fixing carbon dioxide is present; carbon dioxide is liberated, and the amount liberated can be determined previously by standardizing the charged alkaline water in respect to its carbon dioxide content; it is as simple a procedure as the old method of pyrogalllic acid and caustic alkali; the method grows strict anaerobes and type 1 partial tension strains better than any previously devised method, even replacement with gases.

The result of the application of this method is shown in the following experiments:

EXPERIMENTS

Exper. 1.—*B. welchii* from an old stock culture was inoculated thinly on several tubes of dextrose ascites agar, the slants having the water of condensation removed and being previously dried in an incubator. These slants were incubated, one aerobic, another anaerobic, as produced by alkaline pyrogalllic, and the other under carbon dioxide charged alkaline pyrogalllic. After 24 hours' incubation, growth had occurred only in the tube which was placed under anaerobic conditions as produced by carbon dioxide charged alkaline pyrogalllic (fig. 1).

Exper. 2.—Three dextrose-ascites-agar slants were inoculated from an old culture of *B. tctani* that contained only spores. They were incubated, respectively, aerobic, anaerobic, as produced by alkaline pyrogalllic, and anaerobic, as produced by carbon dioxide charged alkaline pyrogalllic. After 48 hours' incubation, growth had occurred only in the tube made anaerobic by pyrogalllic acid and carbon dioxide charged alkali solution. After 80 hours' incubation, a slight growth had occurred in the tube made anaerobic by pyrogalllic acid and caustic alkali (fig. 2).

Exper. 3.—Sputum from a case of chronic bronchitis was washed thoroughly with salt solution, then inoculated on several slants of dextrose ascites agar. These were incubated, respectively; aerobic, partial tension, anaerobic as produced by caustic alkali and pyrogalllic acid, and anaerobic as produced by pyrogalllic acid and charged carbon dioxide alkaline water. After 16 hours' incubation, there was a slight growth in the aerobic, a fair growth in the partial tension, no growth in the anaerobic culture as produced by caustic alkali and pyrogalllic acid and a heavy growth in the anaerobic culture as produced by pyrogalllic acid and carbon dioxide charged alkaline solution (fig. 3).

Exper. 4.—Two tubes of dextrose-ascites agar were thinly inoculated from an old stock culture of *B. botulinus*; one was incubated under the old pyrogalllic acid and caustic alkali method, while the other was incubated under anaerobic conditions as produced by the charged carbon dioxide alkaline solution. Growth appeared earlier and with greater luxuriance under the new anaerobic method than under the older method.

Exper. 5.—A stock culture of staphylococcus was thinly seeded on several tubes of dextrose-ascites agar. The tubes were incubated under the various gaseous conditions, with results that a heavy growth appeared in 24 hours, aerobically, partial tension and anaerobic as produced by pyrogalllic acid and carbon dioxide charged alkaline water, but only a slight growth under pyrogalllic acid and caustic alkali.

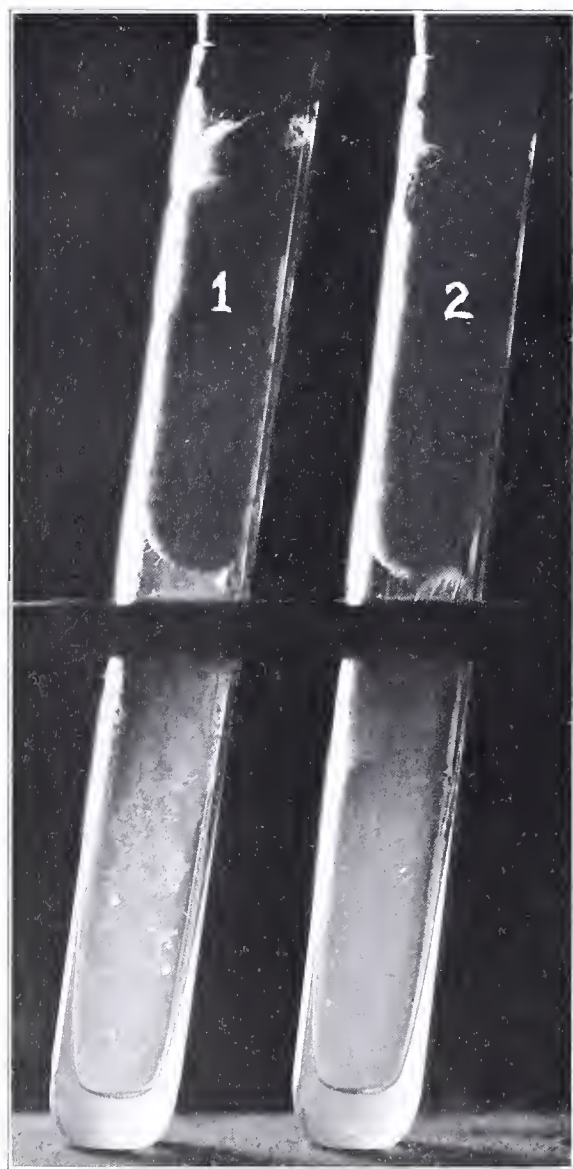


Fig. 2.—Growth of *B. tetani*, tube 1, anaerobic by pyrogalllic acid and carbon dioxide charged alkaline water; tube 2, anaerobic by pyrogalllic acid and caustic alkali.



Fig. 3.—Growth of bacteria from purulent secretion, tube 1 aerobic; tube 2, partial tension; tube 3, anaerobic by pyrogalllic acid and carbon dioxide charged alkaline water; tube 4, anaerobic by pyrogalllic acid and caustic alkali.

DISCUSSION AND SUMMARY

The foregoing experiments show that carbon dioxide is necessary for anaerobic growth of bacteria in many, if not in all, instances. Tests with *B. welchii* and *B. tetani*, *B. botulinus*, anaerobes from purulent secretions, and staphylococci have demonstrated that anaerobic growth of the bacteria will be delayed or entirely prevented in the absence of carbon dioxide if thinly seeded on the mediums.

The ordinary method of absorbing oxygen with pyrogalllic acid and caustic alkali is modified by using pyrogalllic acid and 10% sodium bicarbonate dissolved in water highly charged with carbon dioxide. This method not only removes oxygen, but also liberates carbon dioxide into the test tubes. It will be found by the use of this method that many strains can be cultivated which heretofore have been missed. It is again shown and emphasized by experimental evidence that anaerobic life is more than growth without oxygen, carbon dioxide being an essential factor.

THE IDENTIFICATION OF THE ORGANISM OF ROCKY MOUNTAIN SPOTTED FEVER IN THE BLOOD

CHARLES L. CONNOR

From the Department of Pathology, Harvard Medical School, Boston

The organism of Rocky Mountain spotted fever has been exceedingly difficult to find in the blood of man or animals suffering from the disease. Ricketts¹ saw it when he first studied the disease, and Wolbach has seen it in thick blood smears in endothelial cells. While the serum has been known to contain the infectious agent, it cannot be seen in ordinary preparations. For this reason, the form of the organism (*Dermacentroxenus rickettsi*) in the cell-free serum has been thought to be different from that found in cells and in tissues. However, I have been able to demonstrate the organism, using a method almost similar to that employed by Ricketts.

Ricketts added immune serum to infected serum in varying dilutions, and after centrifugation for 10 hours found the "diplobacillus" in the residue. I have been unable to prove that immune serum has any agglutinating action on the organism in the serum, and so have not added immune serum. Using every aseptic precaution, blood is taken from a guinea-pig 6 days after inoculation with spotted fever blood, the serum separated by centrifugation and then diluted with about 5 times its volume of 0.85% salt solution. Centrifugation at about 2,000 revolutions per minute for at least 6 hours is necessary to throw down the organisms. By staining smears of the residue with Giemsa's stain for more than 4 hours, and differentiating with 95% alcohol for 30 seconds, a few of the organisms may be seen. The smears are first fixed in absolute alcohol, and may be stained over night with a dilute stain. The organisms, even after this procedure, are few in number, but do not differ from the forms in tick and animal tissues as described by Wolbach,² and later by Nicholson.³ They stain pale blue, and have a shadowy outline, and not the clear-cut border of most bacteria. The most common form is the lanceolate diplobacillary form, the ends much more pointed than the diplococcus of pneumonia, and with the flat ends

Received for publication, Aug. 16, 1924.

¹ Ricketts, H. T.: Contributions to Medical Science, 1911.

² Jour. Med. Res., 1919, 41, p. 1.

³ Jour. Exper. Med., 1923, 37, p. 221.

apparently joined by a nonstaining material. Bacillary forms are numerous, but coccoid forms cannot be recognized. The bacilli may be solid or may contain one or two chromatoid bodies. The only variation noted is in size; they may be somewhat larger than when seen in tissue. Figure 1 shows a drawing of about 6 oil immersion fields. This shows the small numbers of them found, but is characteristic of their appearance whenever seen.

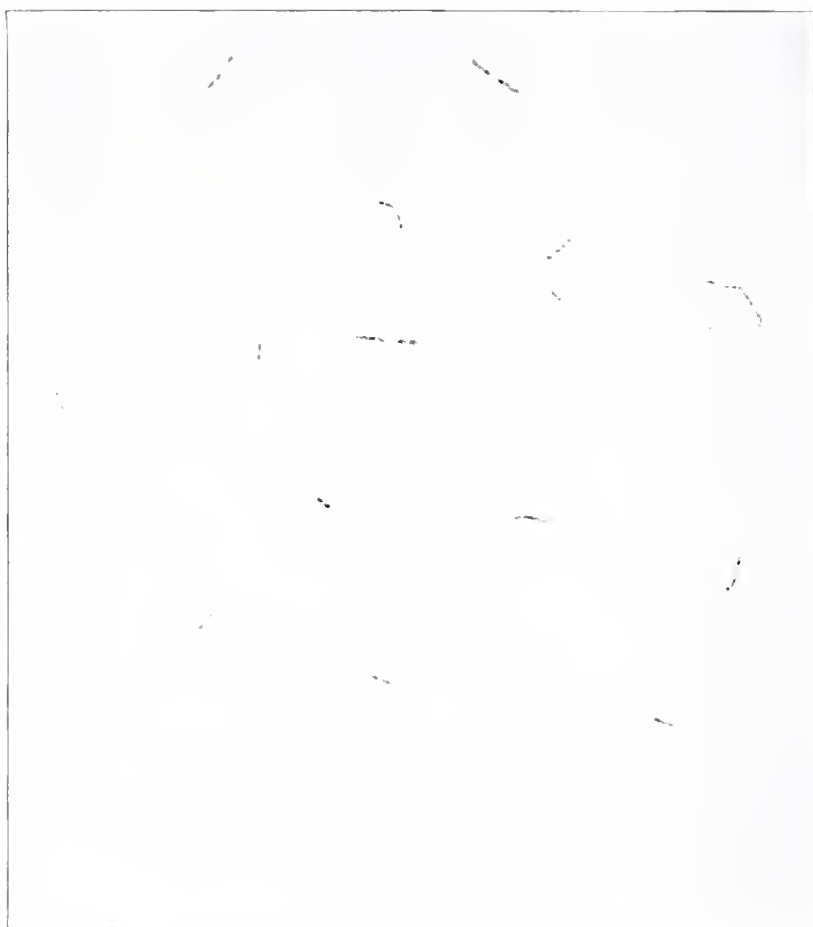


Fig. 1.—Camera lucida drawing at about 1,600 diameters of *Dermacentroxenus rickettsi* as it appears in cell-free serum.

A second method of demonstrating them in whole blood is by dilution with distilled water about 1:5, and prolonged centrifugation. Thick smears may be made of the decolorized residue, and when stained with Giemsa's stain, these show a few of the organisms, mostly bacillary forms in the smears I have made, but having a characteristic diplo-

bacillary arrangement. These bacilli have rounded ends, not tapered, and are seen in both figures 1 and 2.

The third and most reliable method of demonstrating them has been by intraperitoneal inoculation of a normal guinea-pig. In 24 to 48 hours the animal is killed, and smears are made from the peritoneal exudate. Here they are seen both inside and outside the endothelial leukocytes, but most of them within the cells. This method has been successful

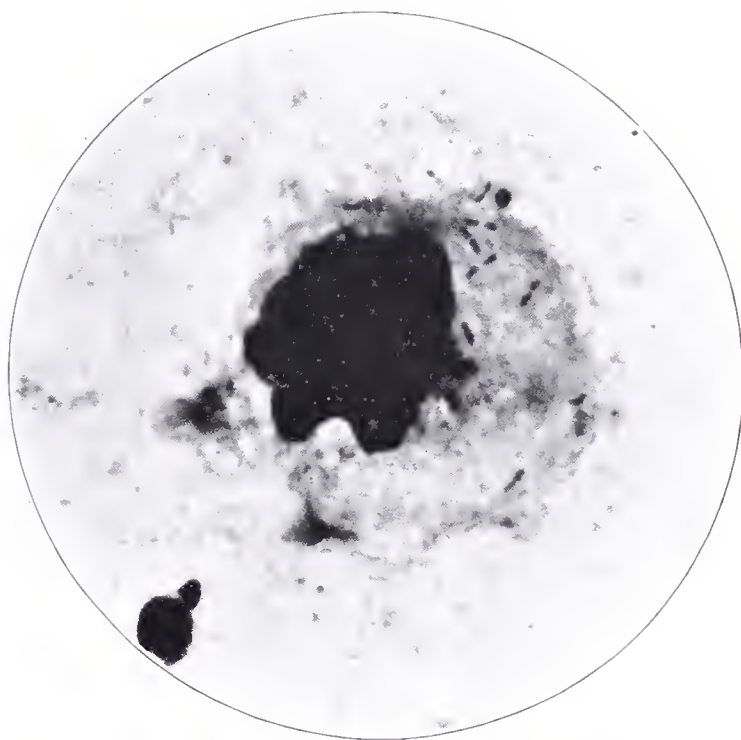


Fig. 2.—*Dermacentroxenus rickettsi* in an endothelial leukocyte from the peritoneal exudate of a guinea-pig 48 hours after inoculation with spotted fever blood. Photomicrograph at 2,000 diameters.

in over half the attempts made. Smears made from a few drops of exudate secured by puncture of the belly wall with a capillary pipet have shown the organism only once, and that after long search. Thick smears are necessary, and commonly about 10 are made. Three or four of this number will show them after less than half an hour's search. They may be stained also by Goodpasture's phenol-anilin-fuchsin or Löffler's methylene blue, but are gram-negative. Figure 2 shows a cell containing organisms.

Control work has consisted of attempted cultivation of organisms from peritoneal exudates and the residue of centrifugated specimens. When contamination has occurred, there has been no difficulty in recognizing the contaminating organism. Most of the cultures have been negative in cases in which the spotted fever organisms were found. Peritoneal exudates and residues were then injected into guinea-pigs, and in all cases caused spotted fever. A negative culture with a positive inoculation test is considered adequate as control.

SUMMARY

The organism of Rocky Mountain spotted fever may be found in the blood and cell-free serum of infected guinea-pigs by dilution and prolonged centrifugation. Staining for more than 4 hours in Giemsa's stain, and differentiation in 95% alcohol seem necessary to bring it out properly.

The organism may be found by recovering infected blood from the peritoneum of a normal guinea-pig 2 days after intraperitoneal inoculation.

The fact that it has been found in the blood and serum does not completely dispense with speculation as to the real form of the organism in this situation. The small numbers found may be present from broken-down cells, and be quite accidental and transient. The unusual virulence of the blood (0.001 c.c. often being infectious) seems somewhat incompatible with the exceedingly small number of organisms found.

OBSERVATIONS ON CHANCROIDAL INFECTION

CLARENCE C. SÆLHOF

From the John McCormick Institute for Infectious Diseases, Chicago

Perhaps the first to distinguish and assign venereal contagion as the cause of chancroid, bubo, and phagedenic ulcer was Guglielmo Salicetti or Saliceto (1216-1277). From then little progress was made until John Hunter (1728-1793) differentiated clearly between hard (hunterian) chancre and chancroidal ulcer. In 1889, Ducrey¹ announced the discovery of a bacillus cultivated from soft chancre, and Unna,² in 1892, confirming the findings of Ducrey, emphasized its marked tendency to chain formation. Nicolle,³ in 1893, and Cheimisse,⁴ in 1894, described at length the morphology of the organism and its culture characteristics in various mediums.

Bezancon, Griffin and LeSourd⁵ advocate the use of agar and rabbit blood for cultivation. They describe their cultures as follows:

On the surface of the inoculated tubes, one sees appearing after 24 hours' incubation at 37 C. the round colonies, white and shining, which do not attain their complete development until after 48 hours, and they are then opaque, grayish, and are 1 to 2 mm. in diameter.

Taking upon a platinum loop, staining with a solution of carbol gentian violet or methylene blue, one sees under the microscope the bacillus which has the exact morphological aspect of grouping described by Ducrey, Unna and Nicolle. They are arranged in chain formation, retaining the stain at their extremities, colorless in the middle part, and do not retain the Gram stain.

Tomaszewski,⁶ systematically searching for the bacillus in a series of cases and using principally rabbit blood agar for isolation, obtained 40% positive results from buboes. Gallia⁷ concluded, from his extensive experiments, that antibody formation occurred in persons affected with soft chancre.

The first properly controlled and successful animal inoculation was recorded by Nicolle,³ who obtained two chancroids in the frontal region

Received for publication, Oct. 1, 1924.

¹ Ann. de dermat. et syph., 1890, 1, p. 56.

² Monatsh. f. prakt. Dermat., 1892, 14, p. 485.

³ Méd. mod., 1893, 4, p. 735.

⁴ Ann. de dermat. et syph., 1894, 5, p. 277.

⁵ Ibid., 1901, 2, p. 1.

⁶ Arch. f. Dermat. u. Syph., 1904, 71, p. 113.

⁷ Ann. de mal. ven., 1907, 2, p. 827.

of the scalp of monkey (*M. rhesus*). The failure of experimenters to obtain satisfactory inoculation in animals is noticeable. In 1894, Straus⁸ reported that a secondary invading organism was necessary to make the inoculation of Ducrey bacillus successful and his work received confirmation by Muric,⁹ Robin,⁹ Mannius⁹ and Ducrey.⁹

My work was undertaken (*a*) to find a method that would make the bacteriologic diagnosis of chancroidal infection accurate and fairly rapid, (*b*) to determine by serologic reactions whether there are different strains of the organism, and (*c*) to study other immune reactions.

The method used in the isolation and the cultivation of the bacillus of Ducrey is essentially that of Teague and Deibert¹⁰ modified as follows: A rabbit was bled from the heart with a sterile 20 c.c. syringe, and 1 c.c. of blood was distributed in sterile test tubes which were then slanted and the serum allowed to exude from the clot and collect in the bottom of the tube. This clotted blood was kept in an icebox for 6 days before being used.

Pieces of No. 20 gauge steel wire were cut into 2 inch lengths, and at one end a small loop was bent, these loops being as nearly constant in size as possible. A loop, held by tissue forceps, was heated to redness in the Bunsen flame and allowed to cool. Inoculum was obtained by rubbing the base of the ulcer or the ulcerated area until serum exuded, at which time the entire loop was dropped into a tube containing the rabbit serum. Four tubes were inoculated from each ulcer and incubated for 24 hours at 37 C. Serum from tubes containing gram-negative streptobacilli was streaked by gradient dilution on the surface of plates of freshly prepared ascites-phosphate agar containing 5% sheep corpuscles, and the plates incubated from 24 to 48 hours. It is imperative for successful cultivation that the temperature is kept constant at 37 C., that abundant moisture is present in the incubator, and that the surface of the agar is moist and not too stiff.

Plates were examined at 24 and 48 hour intervals, and the chief cultural characteristics of the Ducrey bacillus were: The colonies appeared small, round, slightly elevated, and had a mucoid appearance; they were pinpoint in size and produced no visible change in the medium. In 48 hours the colonies were quite characteristic, slightly elevated, round, about 0.25 mm. in diameter, and translucent with a slight zone of incomplete hemolysis. This hemolysis became marked on the 3rd or 4th day. The hemolytic character of the Ducrey colonies escaped the attention of earlier workers, except Teague and Deibert, who showed that there is a definite zone of hemolysis in 3 or 4 days. Later the colonies became whitish or milky, and acquired a metallic refractile sheen.

The shape of the organism varies: It is a bacillus, 0.6 to 1.5 microns in length and 0.3 to 0.6 micron in width; chains may consist of 3 to 25 bacilli; occasionally apparent constriction in the middle of the bacillus is discernible so that it appears coccoid in form. Ascites glucose broth gives excellent chain formation.

⁸ Ann. de dermat. et syph., 1895, 6, p. 9.

⁹ Quoted on authority of J. L. Petit, Historique du chancre mou., 1913.

¹⁰ Jour. Urol., 1920, 4, p. 543.

A slight deviation from the conditions of incubation just described causes various types of involution forms to appear. This phase has been well described by Teague and Deibert, who suggest that the appearance of large numbers of involution forms in rabbit blood heated to 64 C. may serve as an aid to the identification of freshly isolated strains of the bacillus, just as involution forms on salt agar are helpful in the diagnosis of *B. pestis*. My own observations have confirmed this phenomenon, although I would not ascribe much value to it as a diagnostic procedure because of its variability.

Growth of the freshly isolated organism on the common laboratory mediums was not obtained. It was found that the medium must be enriched with various body fluids and that the organism grows most luxuriantly in a medium containing both ascites fluid and blood cells.

Basic aniline dyes inhibit the growth of gram-positive bacteria more strongly than gram-negative bacteria. It was assumed that the use of such dyes might prove of value in isolation and cultivation, since the most contaminating organisms in chancroidal infection are gram-positive cocci and diphtheroid bacilli. However, it was found that the concentration of the dye had to be so high that not only the gram-positive organisms were inhibited, but also the Ducrey bacillus.

The Ducrey bacillus was isolated and cultivated from both male and female patients, irrespective of the duration of the lesions. They were cultivated, in the manner described, from 122 venereal ulcers—117 in males and 5 in females. The incidence of the organisms obtained is summarized in table 1. Cultures were made repeatedly of some ulcers.

TABLE 1
PERCENTAGE INCIDENCE OF BACTERIA OBTAINED BY CULTURE FROM VENEREAL ULCERS

Clinical Diagnosis	Sex	Cases	Ducrey Bacilli	Staph. albus (hemo-lytic)	Staph. albus (nonhe-molytic)	Staph. aureus	Strep-tococci (nonhe-molytic)	Diph-theroid Bacilli
Chaneroid.....	M	64	64	17.5	12.5	1.7	10.92
Phimosis.....	M	7	..	14.28	49.0	28.6	14.2
Buboes.....	M	8	25	87.5	13.5
Circumcision ulcer...	M	1	100.0
Herpetic ulceration...	M	2	50.0	50.0
Chanere.....	M	20	10.0	5.0
Chaneroid.....	F	2	50	50.0
Buboes.....	F	3	100.0

As shown, 44 strains of Ducrey bacilli were isolated, 42 from clinical instances of chancroidal infection, all in males except one. The remaining 2 strains were isolated from buboes. I was able to isolate the

organism in only 65% of clinical chancroids. Teague and Deibert mention that with their method they might be able to isolate the organism in about 90%. The recovery of the organism from buboes is exceedingly difficult, due to overgrowth of secondary contaminating organisms, mainly *Staphylococcus albus*.

Brams¹¹ reports that in 5 instances from smegma from 30 selected men with phimotic foreskins, 27 of whom were negroes, he isolated a gram-negative streptobacillus, with the morphologic and cultural characteristics of the Ducrey bacillus. He concludes that:

Persons apparently normal may be carriers of the Ducrey bacillus. A person does not necessarily acquire the disease during sexual intercourse with an infected partner. The so-called mixed infection does not mean that the person was exposed to a partner who had both chancre and chancroid.

Cultures were made from 26 persons like those described by Brams,¹¹ and I was able, in two instances, to isolate a gram-negative streptobacillus. Both strains appeared markedly atypical in comparison to strains isolated from chancroidal infection. They were pleomorphic, varied in their cultural reactions, and, particularly in fluid medium, tended markedly to resemble streptococci. A small hemolytic zone was noticed about the colonies similar to that observed surrounding the colonies of the Ducrey bacillus. They did not tend to produce agglutinins in rabbits, and the opsonic index of the patients' serum was extremely variable.

The presence of many colonies of contaminating organisms had a marked deleterious effect on the growth of the Ducrey bacillus. It was found that in those instances in which a great number of contaminating colonies were present and abundant, it was almost impossible to isolate or cultivate the Ducrey bacillus. Whether this growth inhibition is due to a substance formed in the growth of the contaminating colonies with subsequent dissemination throughout the medium or whether it is due to direct changes in the medium itself, I am unable to state.

A noticeable feature was the large number of hemolytic staphylococcic colonies that were observed in association with the Ducrey bacillus in chancroidal infection. The cultural characteristics of these hemolytic staphylococci were studied in some detail, and the results agreed with those obtained by Julianelle.¹² It was found in a general way that *Staphylococcus aureus* reveals hemolysis earlier than *Staphylo-*

¹¹ Jour. Am. Med. Assn., 1924, 82, p. 1166.

¹² Jour. Infect. Dis., 1922, 31, p. 256.

coccus albus and also that strains virulent for rabbits show hemolysis earlier.

The freshly isolated strains of Ducrey bacilli were subjected to agglutination tests in order to determine whether different groupings might be obtained. The tendency of the Ducrey bacillus to grow in long chain formation and on suspension to form clumps spontaneously caused considerable difficulty. The following procedure was used to obtain a complete and even suspension. The washings in salt solution from 6 or 8 plates, 48 hours old, were placed in the small globe of a ball mill, which was then allowed to run continuously for 48 hours. At the end of this period the emulsion was removed, centrifuged slowly to remove clumps of organisms, and the supernatant fluid diluted to a suitable suspension.

Rabbit serum was used exclusively. Rabbits were immunized by injecting intravenously, at different intervals, both large and small amounts of suspensions of the organisms. The titer of the serum obtained by bleeding from the ear vein at varying intervals was determined. The results were unsatisfactory and not constant. Occasionally the organisms were agglutinated readily with normal rabbit serum. No evidence of serologic grouping for the strains used was found. The results are essentially in accord with those obtained by Teague and Deibert.¹³

It seemed advisable to determine the opsonic index and the point of opsonic extinction. The 48-hour growth of organisms was suspended in salt solution. Normal human leukocytes, collected in 0.2% sodium citrate solution, were used in all experiments. The mixtures of serum, leukocytes, and suspension of bacilli, in equal parts, were incubated at 37 C. for 20 minutes and smears stained by the Giemsa method. One hundred polymorphonuclear leukocytes were counted, and the number of bacteria and cells taking part in phagocytosis were noted. It was found that the point of opsonic extinction of the serum of chancroidal patients varied from 1:48 to 1:384, the average point of extinction being 1:96 for these strains of Ducrey bacilli, the average point of extinction for normal serum being 1:16. No definite grouping was obtained by this method.

Bruch and Ito¹⁴ prepared an autogenous vaccine against chancroids, which they claim gave good results in a short period of time.

¹³ Jour. Med. Res., 1922, 43, p. 61.

¹⁴ Arch. f. Dermat. u. Syph., 1913, 116, p. 341.

Stümpke¹⁵ prepared a vaccine in the following manner: The Ducrey bacillus was grown for 48 hours on blood agar, suspended in salt solution and heated to 65 C. for one-half hour. A small amount of phenol as a preservative was added. The finished product contained 10,000,000 organisms per c c. Intramuscular doses of 0.1 to 5 c c. were given with variable results in a few cases. Reenstierna¹⁶ gave intravenous injections of vaccine in increasing doses. He states that the serum of these patients gave good complement fixation, and from 3 to 4 injections usually sufficed for a clinical cure. Later,¹⁷ he reports excellent results from the subcutaneous injections of an antiserum for the Ducrey bacillus. This antiserum was obtained from sheep. It contained active antibodies and also a temperature-raising agent (killed typhoid bacilli) to enhance its therapeutic efficiency. In some of the most striking cases which he records the patients were not treated alone with the serum, but in conjunction with other local measures, such as surgical cleanliness, copper sulphate applications, etc.

For orientation I treated with vaccine 4 patients with chancroids, 3 of recent origin, the other a chronic serpiginous ulceration. The vaccine had been prepared according to Stümpke's method. The results were apparently negative, both as regards the rapidity of healing or the disappearance of the lesions. Two cases of chancroidal infection as control healed as readily under other methods of treatment as those receiving vaccine injections. An occasional foreign protein reaction was elicited by intravenous injections.

Intravenous injections of small and large doses of Ducrey bacilli were given to healthy mature sheep at varying intervals. At regular periods, blood was withdrawn for serum and the antibody titer determined. At highest titer obtained, the animals were bled, the serum pooled and filtered through Berkefeld V filters and sufficient preservative added. Two patients with chancroid were given large doses of this antiserum intravenously, the lesion in one instance disappearing 3 days after injection of 30 c c., with no local treatment; the lesion in the second instance responding 4 days after 2 intravenous injections of 25 and 30 c c., respectively, at 2-day intervals. The lack of a sufficient number of cases allows no conclusions to be drawn.

The technic to determine the bactericidal index for the Ducrey bacillus was as follows: Ten small test tubes were placed in a rack, and in each was

¹⁵ Deutsch. med. Wchnschr., 1921, 47, p. 1331.

¹⁶ München. med. Wchnschr., 1920, 31, p. 895.

¹⁷ Arch. d. l'Inst. Pasteur d. Tunis, 1923, 12, p. 273.

placed an equal volume of ascites broth. A predetermined amount of the selected drugs—argyrol, silver nitrate, neutral acriflavine and mercurochrome—was added to each tube in percentage dilution as described in table 2. To each tube was added an equal amount of 24-hour suspension of freshly isolated Ducrey bacillus. As each standard amount of organism was added, the tubes were immediately shaken and the time noted. Using a standard platinum wire loop, equal amounts of fluid were withdrawn at regular intervals and plated on freshly prepared plates of ascites blood phosphate agar, with excess of water of condensation on the surface. The water of condensation was immediately mixed with the loopful of organisms to dilute the infinitesimal amount of antiseptic carried over, and the plate surface streaked. Incubation for 24 hours was allowed, at which time an accurate relative count of the number of colonies present, if the total count was above 150, was made. To be within limits of error, these experiments were repeated 4 times, and table 2 represents the averages obtained.

TABLE 2
RELATIVE EFFICIENCY OF ANTISEPTICS AGAINST DUCREY BACILLUS

Percent. Anti- septic Dose	Argyrol: Minutes after Dose					Silver Nitrate: Minutes after Dose					Neutral Acriflavine: Minutes after Dose					Mercurochrome: Minutes after Dose				
	0	5	10	30	60	0	5	10	30	60	0	5	10	30	60	0	5	10	30	60
2.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.25	2	0	0	0	0	0	0	0	0	0	8	0	0	0	0	0	0	0	0	0
0.125	7	0	0	0	0	2	0	0	0	0	6	2	0	0	0	0	0	0	0	0
0.0625	11	1	0	0	0	2	0	0	0	0	19	10	2	0	0	0	0	0	0	0
0.03125	29	6	0	0	0	9	1	0	0	0	35	16	8	0	0	2	0	0	0	0
0.015625	116	11	2	0	0	31	18	5	0	0	185	35	25	2	0	9	2	0	0	0
0.0078125	125	18	7	1	0	118	36	0	0	0	600	350	100	12	1	200	58	32	7	0
Control	700	600	700	700	700	700	600	700	700	700	700	600	700	700	700	700	600	700	700	700

The numbers refer to the number of colonies per plate after exposure to drug and subsequent plating. Incubation for 24 hours. Colonies greater than 150 approximated only.

As will be noticed, the results varied. The least efficacious drugs apparently were argyrol and neutral acriflavine; the most efficient, silver nitrate and mercurochrome. While these in vitro tests may be essentially correct, in vivo the index probably would not be so high, as the action of the drug may be inhibited by secretions and by chemical changes, etc.

ANIMAL INOCULATION

Efforts at experimental production of soft chancre by inoculation of laboratory animals with the material obtained from active lesions of chancroids or pure culture of the Ducrey bacillus have given inconstant results. In 1889, Nicolle¹⁸ published his results on the experimental production of soft chancre, which proved to be of positive character. In a review on the production of experimental soft chancres in the rabbit,

¹⁸ Compt. rend. Soc. de biol., 1893, 51, p. 778.

by Terebenski,¹⁹ rabbits were inoculated intradermally with secretion obtained from ulcerating chancroids, and small crust covered eruptions were obtained. On removal of the crust, the condition, both clinically and microscopically, was similar to soft chancre in man. Microscopically, the adjacent lymph vessels were markedly implicated, and the organisms were observed in these channels before being discovered in the lesions. Reenstierna²⁰ states that the inoculation of the Ducrey bacillus into the skin of monkeys gives rise to the development of pustules, which largely correspond to soft chancre in man. This occurs whether the inoculum comes from the secretion of a sore or a pure culture of bacilli. He further states that similar experiments on other species of animals gave exclusively negative results. Reenstierna states that Fontana succeeded in producing in rabbits a keratitis followed by ulceration, the inoculum being the secretion of the sores and pure cultures of the organism. Of 25 inoculations, 7 were positive; reinfection was obtained, but with a marked decrease of intensity. Further transmission of the experimental keratitis was obtained by implantation of small pieces of infected cornea into the anterior chamber of the eyes of rabbits. In the affected areas, demonstration of the bacillus was made with the microscope. Inoculation of segments of experimentally infected cornea produced soft chancre in man.

Reenstierna, using a pure strain of organism obtained by human auto-inoculation, punctured the scrotum of 10 male rabbits with a scalpel dipped in an emulsion of Ducrey bacilli, of the 14th generation. Of 10 animals used, 6 were negative, while 4 revealed small red spots at the site of puncture. Two days later, pustules formed with no tendency to break or to form ulcers. When the crust was removed, a shallow sore was revealed, with an undermined, irregularly defined margin. Direct smear or culture of these sores failed to reveal the bacilli. Inoculation of a man with the product of the lesions of these rabbits produced typical soft chancre in 2 of 3 injections. Direct smear and culture of the human lesions revealed the organism. On section, the rabbit testicle revealed what he states to be considered the histopathology of soft chancre with bacilli greatly scattered, both intracellularly and extracellularly.

In these experiments, healthy young male rabbits were used, and various strains of recently isolated Ducrey bacilli. Subcutaneous inocu-

¹⁹ Russki Wratsch, 1915, 32, p. 795.

²⁰ Urol. & Cutan. Rev., 1921, 25, p. 332.

lation gave negative results in 11 of 12 animals, one producing a papule at the site of inoculation. Eventually this became pustular, with negative smear and culture preparations. A concurrent respiratory infection by the *B. bipolaris* had lowered resistance sufficient to allow secondary infection. Inoculation of pure cultures directly into the inguinal lymph glands of 10 animals showed no results. Scarification of the skin plus implantation of an emulsion of the organism directly on the abraded surface, with the exception of local inflammatory results, was negative.

Using a *Macacus rhesus* monkey, scarification of the glans penis with subsequent exposure of the irritated surface to an emulsion of Ducrey bacilli produced no lesion. Intradermal injection of 0.3 c.c. of pure cultures of Ducrey bacilli into the inguinal region produced lesions passing through the stage of papule to pustule without breaking through the crust. Removal of the crust revealed a shallow, undermined ulcer, with a dirty, necrotic base. Smears of the discharge stained by the method of Gram revealed a gram-negative streptobacillus. Cultures remained sterile; no buboes were evident. It has been relatively easy to produce in a small number of instances an experimental soft chancre in the monkey, but in rabbits, no lesions have been produced.

DISCUSSION

From the literature, the cultural characteristics of the Ducrey bacillus appeared varied. The growth requirements of the organism are highly selective, and unless special modifications of medium are used in isolation, failure to obtain the organism is quite constant. The application of cultural diagnosis was first discussed by Moore,²¹ who made cultures in 55 cases on blood serum agar and obtained positive cultures in only 5 instances. He stated: "Obviously, therefore, these methods of microscopic and cultural diagnosis are not to be relied upon." Moore's failure to obtain satisfactory results probably was due to the fact that his culture medium was not suited to the growth of the Ducrey bacillus. As pointed out by Teague and Deibert and stated previously in this article, great care must be used in the choice of a medium favorable to the organism. A constant temperature plus excessive moisture are also required. These conditions are likewise favorable to the growth of contaminating organisms.

²¹ Jour. Urol., 1920, 4, p. 169.

The involution forms of the organism are marked. It has been suggested that these involution stages may be of value in confirming the presence of the Ducrey bacillus. From observation, these involution forms are extremely variable, but with some experience they might possibly be of assistance in establishing the identity of the organism as a diagnostic procedure in routine practice.

Positive isolation and cultivation of the Ducrey bacillus was obtained in only 65% of clinical chancroids, a much smaller figure than that of Teague and Deibert who state that "Since more than 50% of all sores cultured by us were positive for the Ducrey bacillus, and since most of the negative cases showed no clinical evidence of chancroid, it follows that a diagnosis of chancroid can be made by means of the cultural method in a very large percentage of cases—probably above 90%." The isolation of a streptobacillus from normal persons having the characteristics described by Brams, was obtained in only 2 of 26 instances. Brams states that normal persons may be carriers of the Ducrey bacillus. Pijper²² states that "The bacillus of Unna-Ducrey is at present universally acknowledged as the cause of soft sore; it is only mentioned in passing that a strikingly similar organism, the streptobacillus urethrae, has been cultivated by Pfeiffer from the normal human urethra." He accepts the following statement in the report of the British Medical Research Committee: "The Committee²³ finds no sufficient evidence that what is clinically known as soft chancre or soft sore is a specific disease induced by a single species of microorganisms." From facts cited, it appears that the bacteriologic status of chancroids is still indefinite. This discrepancy in facts may be due to the extreme difficulty in the isolation and cultivation of the bacillus from contaminating organisms and the varied vitality of the organism itself.

The extremely variable results that were observed in my serologic work are quite in accord with those cited by Teague and Deibert. The titers obtained varied greatly. The lack of constant results makes it impossible to classify different strains of the organisms into groups, or to assist materially in the classification of the two strains isolated from normal persons. Whether the negative results are due to lack of antibodies in the immune serum or to the inagglutinability of the bacteria or both, is still undetermined. The varied results obtained in the deter-

²² Med. Jour. South Africa, 1920-21, 16, p. 89.

²³ Med. Res. Committee, Special Rep. Ser. No. 19, 1918, p. 47.

mination of the opsonic index may be related in some manner to the variability in agglutinin titer. No definite grouping could be determined from results obtained.

The use of vaccines in few instances gave practically negative results. Equally good results were obtained by local treatment.

The bactericidal index was determined against 4 commonly used drugs. As will be observed, 2 silver compounds were used, the best sterilizing effect being obtained by silver nitrate. Two dye compounds were used, one being combined with mercury. The mercury dye preparation (mercurochrome) in vitro gave a most efficient sterilizing index.

The efforts at experimental production of soft chancre in rabbits gave consistently negative results. Intradermal inoculations of monkeys, however, produced a crusted lesion, and on removal of the crust, a shallow undermined ulcer with a necrotic base was exposed, smear preparations of which revealed the Ducrey bacillus.

SUMMARY

Isolation and cultivation of the Ducrey bacillus was successful in 65% of clinical chancroids.

Failure of isolation may be due to use of improper mediums, lack of constant temperature and sufficient moisture.

A zone of hemolysis was evident about the colonies after from 2 to 4 days' incubation.

Involution forms of the organism are constant, and with some experience, might possibly be of assistance in establishing the identity of the organism in routine practice.

Two strains of a streptobacillus having the morphologic and cultural characteristics of the Ducrey bacillus were isolated from the smegma of normal persons.

The lack of uniform results makes it impossible to classify the Ducrey bacillus by serologic methods into definite groups or to assist materially in the classification of strains obtained from normal persons.

In a limited number of instances, vaccines were apparently of no therapeutic value in the treatment of chancroidal infection.

The bactericidal index was determined against 4 drugs; 2 silver compounds and 2 dye compounds, 1 being combined with mercury. While these in vitro tests may be essentially correct, in vivo the index

probably would not be so high, as the action of the drug may be inhibited by secretions, chemical changes, etc.

Production of experimental chancroid in the rabbit gave uniformly negative results. Intradermal inoculation of pure cultures of Ducrey bacillus in the monkey produced a shallow, undermined, crust-covered ulcer, smear preparations of which revealed the Ducrey bacillus.

THE TWORT-D'HERELLE PHENOMENON

THE RESEMBLANCE OF BACTERIOPHAGE TO TOXINS AND FERMENTS

LLOYD ARNOLD AND EMIL WEISS

*From the Department of Bacteriology, Pathology and Preventive Medicine, Loyola University
School of Medicine, Chicago*

D'Herelle¹ believes that the phenomenon of bacteriophagy is produced by a bacteriolytic ferment-like substance secreted by a microbacterium (*Bacteriophagum intestinale* d'Herelle, 1918). If this ultra-microbic parasite of the bacteria is killed, the bacteriophagic substance cannot be regenerated. The following experiment was carried out by d'Herelle.²

Precipitate a culture of antidysentery bacteriophage with alcohol and dissolve the precipitate in a quantity of 0.8% salt solution equal to the original volume of the culture. Mix equal parts of this solution and broth and add *B. dysenteriae* suspension sufficient to give a slight turbidity. As a control, prepare a tube containing an equal volume of bacilli suspended in a medium half broth and half salt solution. Place these tubes in an incubator at 37 C. After 24 hours the control is turbid, the broth containing the lysin is slightly cloudy. Planting on agar from the two tubes gives normal bacillary growths. After 48 hours the control presents the same appearance and agar inoculation gives a perfect growth. The culture containing the lysin is slightly cloudy and inoculation on agar gives only isolated colonies. A count shows that there are 22 times less living bacilli in the last culture than in the control tube. After 3 days the appearance is the same as after 48 hours. After 4 days the bacteria begin to develop a resistance to the action of the lysin, the medium becomes cloudy and inoculations on agar again give a film of growth.

D'Herelle interprets the difference in cloudiness of the 2 tubes during the first 3 days as being due to the lysin content of the precipitated bacteriophage culture. After the 3rd day, the bacteria became resistant, and their growth produced a cloudiness equal to that of the control tube. The bacteriophage was killed by the alcohol, and the lysins remaining in the precipitate could not be regenerated. This is an important experiment for d'Herelle's conception of the phenomenon of bacteriophagy.

We have pointed out the similarity between the lysin-antilysin, or the bacteriophage-antibacteriophage reaction and those of toxin-antitoxin as well as those of ferment-antiferment nature.³

Received for publication, Sept. 11, 1924.

¹ *The Bacteriophage*, Baltimore, 1922.

² *Ibid.*, p. 124.

³ *Abstr. of Bacteriol.*, 1924, 8, p. 28; *Jour. Infect. Dis.*, 1924, 34, p. 317; *ibid.*, 1924, 35, p. 505.

D'Herelle assumed that the substances causing the depressions of growth in the tube containing the alcohol-precipitated bacteriophage, as compared to the control tube, were lysins produced by Bacteriophage intestinale. He does not mention irregular or lysogenic dysenterial colonies on the subcultures made on agar. The antidysentery bacteriophage was precipitated with 9 parts of alcohol, left standing for 48 hours, the supernatant fluid decanted and the precipitate redissolved in salt solution. It is well known that many ferments are precipitated with alcohol, but the prolonged action of alcohol acts injuriously on the ferments and also makes them insoluble.⁴ We have repeated d'Herelle's experiment. The time of contact between the bacteriophage and alcohol was varied from 3 hours to 7 days. The same concentration of alcohol was used. The precipitate was redissolved in one half and also in equal amounts of salt solution (0.85%), as compared to the original bacteriophagic substances instead of the broth tube method.⁵ The alcohol precipitate redissolved in salt solution had all of the properties of the original bacteriophage. It produced lysis; this lysis was transmissible and increased in power during transmission through susceptible bacteria. The lytic activity of the alcoholic precipitate was inhibited or neutralized by bacteriophagic antiserum. The precipitate, used as an antigen, produces antilysins that neutralize the lytic activity of the soluble alcoholic precipitate as well as neutralizes the phagic activity of the original bacteriophage.

We found that the fresh, moist precipitate dissolved much better in salt solution than the same precipitate dried to a powder. The shorter the period of time that the bacteriophagic substance is in contact with the alcohol, the stronger is its lytic power. The technic of the preparation of the antilysins from the extract of the alcohol precipitated lysins was the same as described for antilysin production against the original lytic substance.³ The antilytic titer of the rabbit serum was seldom as high as that obtained by the injection of the regular bacteriophage. Bacterial antibodies, agglutinins and precipitins were present in these antisera as in the original antibacteriophagic sera, but their titer was always lower.

We added our redissolved precipitate directly to the surface of dried sterile agar plates, or, if weak, we added the extract to concentrated melted agar (50C.) in the ratio of 1:10 or 1:20, and seeded the plates

⁴ Abstr. of Bacteriol., 1922, 6, p. 268.

⁵ Jour. Lab. & Clin. Med., 1923, 8, p. 720.

with susceptible bacteria and incubated them. In this manner, bacteriophagic activity can be determined that would be entirely missed by d'Herelle's technic. When there was evidence of bacteriolytic activity on such an agar plate, the agar was removed, placed in a large test tube, broken up into small fragments with a sterile glass rod, 10 c.c. of distilled water added, and placed in the icebox over night. The fluid was removed from the agar by filtration, then passed through a Berkefeld candle. This filtrate was added to an agar plate, and the same process of seeding, incubation and extraction, etc., repeated. Table 1 shows the results obtained by this method, using an extract of precipitated bacteriophage after the alcohol was in contact with the precipitates

TABLE 1

THE INFLUENCE OF TIME OF CONTACT WITH ALCOHOL ON THE WEAKENING OF BACTERIOPHAGE

Time Precipitate in Contact with Alcohol	Bacteriophage before Precipitation	Bacteriophage Precipitated with Alcohol and Redissolved in Salt Solution	Transmission of Precipitated and Redissolved Bacteriophage					
			1	2	3	4	5	6
3 hrs.	95-100%	60-70%	70%	70-75%	75-80%	90%	95-100%	100%
2 days		30-40%	55-60%	60-70%	70-75%	75-80%	85-90%	90-100%
1 week		10-15%	25-35%	40-50%	50-60%	60-70%	70-80%	80-90%

for 3 hours, 2 days and 7 days. The bacteriophagic activity is expressed in percentage as explained in previous papers.^{3, 5} It will be noted that 7 days' contact with alcohol decreased the activity of the precipitated lysins 90% ; 3 hours' contact decreased the activity only 35% as compared to the original bacteriophage. Transmission, by extracting agar plates containing a 24-hour growth of lysogenic bacteria, can be shown with increase in potency of the lytic principle. The weaker the lytic agent, the more slowly regeneration takes place.

D'Herelle diluted the extract of the alcoholic precipitate with an equal amount of broth. The alcohol had been in contact with the lytic principle for 48 hours. This diluted and weakened lytic substance in broth was seeded with a susceptible bacterium. Some growth inhibition or retardation was noticed as compared with the control of broth diluted with an equal amount of salt solution alone and inoculated with the

same dose of bacteria. Subcultures on agar showed normal bacterial growths; the tube containing the precipitated lysins gave fewer colonies than the control. After 4 days, both tubes had the same turbidity, and subcultures on agar gave a film of growth. D'Herelle assumes a development of resistance of the bacteria in the precipitate-broth tube, and explains the latent growth as being due to this factor. He probably had some alcohol and many other precipitable growth-inhibitory substances in the salt solution that was added to an equal amount of broth and inoculated with bacteria. The original lysins were weakened by his long extraction method, and they were not present in sufficient quantities to be demonstrable by his technic under the adverse growth condition present in his diluted culture medium.

Bacteriophage treated with 9 parts of alcohol yields a precipitate that is soluble in normal salt solution. This extract has all of the properties of the original bacteriophage. The lytic power of the precipitate can be increased by passage through susceptible bacteria; the antilyns produced after animal inoculation of the extract of the alcoholic precipitate are the same as those produced as a result of bacteriophage injection; heat inactivation is the same for both the precipitated lysins and the original bacteriophagic lysins. We have been unable to substantiate the claim that the active principle causing the phenomenon of bacteriophage is an ultramicroscopic parasite.

ANTIGENIC PROPERTIES OF HEATED BACTERIOPHAGE

Ehrlich ⁶ showed that diphtheria toxins would change under certain experimental conditions so that their toxicity would be decreased, but this altered toxin still possessed the power of neutralizing the same amount of antitoxin as the original unaltered toxin. He explained this as being due to changes in the toxophore group, the haptophore, or antitoxin binding group, remaining unaltered. Such modified toxins, Ehrlich refers to as "toxoids." Myer ⁷ found the same altered toxin reaction in cobra lysins, Neisser and Wechsberg ⁸ in staphylotoxins, and Jacoby ⁹ in ricin.

We heated bacteriophage for 1½ hours at 85 C., and 45 min. at 100 C., and there was no trace of bacteriophagic activity in such heated material. Four rabbits were immunized in the usual manner. The antiserum possessed a lower

⁶ Klin. Jahrb., 1897, 6, p. 299.

⁷ Jour. Path. & Bacteriol., 1900, 6, p. 415.

⁸ Ztschr. f. Hyg. u. Infektionskr., 1901, 36, p. 299.

⁹ Hofmeister's Beiträge, 1902, 1, p. 51.

antibacteriophagic activity than the control unheated bacteriophage or the extract of the alcohol precipitated bacteriophage. Table 2 shows an experiment that illustrates the antilysin content of 1 of the 4 serums. It will be noted that the heated bacteriophage gives a lower titer, but a pronounced reaction. The agglutinins are also decreased in the heated bacteriophagic antiserum. The standard unit of antilyns referred to in this table was explained in a previous paper.

TABLE 2

ANTIBODIES OF HEATED (85-100 C.) PRECIPITATED, REDISSOLVED AND NORMAL BACTERIOPHAGE

Antigens	Antibacteriophage or Antilyns	Agglutinins
Heated bacteriophage.....	0.2 c c. — 1 unit	1:40
Precipitated redissolved bacteriophage.....	0.15 c c. — 1 unit	1:80
Normal bacteriophage.....	0.01 c c. — 1 unit	1:5,120

Active and inactivated bacteriophage were mixed with an equal quantity of antibacteriophagic serum. The bacteriophage was inactivated by heating for 1 hour at 60 C. and for 1 hour at 80 C. These 3 tubes were incubated at 37 C. for 24 hours. One-tenth was then added to the surface of an agar plate, seeded with 1 loop of *B. typhosus*, smeared and inoculated in the usual manner. This experiment was repeated, and 0.05 c c. of active bacteriophage was added to each plate before smearing. Controls were included to show the potency of the active and inactivated bacteriophage against the *B. typhosus*. Table 3 shows the results of this experiment. Bacteriophage heated for 1 hour

TABLE 3

THE NEUTRALIZATION OF ANTIBACTERIOPHAGIC SERUM BY ACTIVE AND INACTIVATED BACTERIOPHAGE

	Bacteriophage 0.05 c c.	Additional Bacteriophage	Results
Antibacteriophagic Serum 159 0.05 c c.	Active	0	Normal growth
	Inactivated at 60 C.	0	Normal growth
	Inactivated at 80 C.	0	Normal growth
	Active	0.05 c c.	50% irregular growth
	Inactivated at 60 C.	0.05 c c.	50% irregular growth
	Inactivated at 80 C.	0.05 c c.	50-55% irregular growth
Controls	Active	0	95-100% irregular growth
	Inactivated at 60 C.	0	70-80% irregular growth
	Inactivated at 80 C.	0	Normal growth

at either 60 C. or 80 C. neutralized the same amount of antibacteriophagic serum as the active unheated bacteriophage. An excess of active bacteriophage added to these mixtures produced the same degree of bacteriophagic activity in all 3 instances.

It might have been claimed that in the first 3 reactions there was a sufficient excess of antibacteriophagic serum above the neutralizing dose to allow normal growth, but this cannot be the case, as the addition of an active bacteriophage produces the same degree of irregularity in

all 3 reactions. The controls show that the active bacteriophage is potent; heating for 1 hour at 60 C. decreases the activity 20 to 30%, and heating for 1 hour at 80 C. destroys the bacteriophage action. We can then destroy the toxic or lytic activity of a bacteriophage by heat, and still this inactivated bacteriophage has the power to neutralize antibacteriophagic serum. In other words, the toxophore group can be destroyed and the haptophore group remain, and this is as active in neutralizing the antiserum as when the toxophore group is present. This modified bacteriophage, comparable to the modified toxins or "toxoids," has been shown in the previous experiment to be antigenically capable of producing antibacteriophagic bodies.

THE CHEMICAL NATURE OF ANTILYSINS

Crawford and Foster¹⁰ have recently reviewed the literature on the chemical nature of antitoxins. The antibodies are usually precipitated with the globulin fraction of the serum, particularly the first globulin or euglobulin fraction. We were interested in ascertaining whether the antibacteriophage could be salted out of the antiserum in the same manner.

Sodium sulphate in the concentrations recommended by Howe¹¹ were used for these experiments. Five c.c. of normal and the same amount of antibacteriophage serum were added to sodium sulphate solutions to make the following concentrations: 13.5, 17.5, 12.5 and 32%. These mixtures were placed in the incubator for from 4 to 6 hours, and filtered through dry, hard filter paper. The filtrates were dialyzed through collodion membranes until the surrounding water gave no appreciable cloud with barium hydroxide; this usually required from 12 to 36 hours' suspension in running water. The contents of each collodion sac were then evaporated by means of an electric fan to the original serum volume. Each of the precipitates was redissolved in salt solution and dialyzed free of the sodium sulphate; the dialysate was evaporated in the same manner to the original serum volume. Care was taken to have the final material in a normal salt solution. We have shown in a previous publication³ that antibacteriophages do not readily pass through a Berkefeld filter. We, therefore, felt that our final concentrated serum fractions could not be sterilized by passing through the Berkefeld candle. We also showed that heating for 1 hour at 60 C. did not materially change the antibacteriophage. We heated each fraction, both precipitate and filtrate, for 1 hour at 60 C. each day for 3 successive days.

The antiphagic activity of each fraction was tested. The surfaces of agar plates were seeded with a standard dose of bacteria and 1 unit of bacteriophage;³ 3, 6 and 9 drops of the various fractions were added to 3 successive

¹⁰ Am. Jour. Pharmacol., 1918, 90, p. 765.

¹¹ Jour. Biol. Chem., 1921, 49, pp. 93 and 109.

plates; after smearing with a sterile, bent glass spreader, plates were inverted and incubated for 24 hours. Controls of bacteriophage, serum precipitated fractions, and bacteria alone were included in each series.

The normal rabbit serum did not show an antibacteriophagic action in any fraction. With the antibacteriophage rabbit serum, the precipitate from fraction 1 (13.5% sodium sulphate) contained strong antibacteriophage; the filtrate from this fraction was negative. All of the precipitates of the other 3 fractions were positive, as all contained the euglobulin fraction. The filtrates of all fractions were negative. In another experiment, we added 10 c. c. of antibacteriophagic serum to a solution of sodium sulphate to make a final 13.5% solution of this salt in the mixture, incubated it for 3 hours and removed the precipitate by filtration. The filtrate was made up to 17.5% with sodium sulphate, the process again repeated and the filtrate made up to 21.5% with sodium sulphate; the precipitate of this fraction was separated from the fluid by filtration. The filtrates, as well as the dissolved precipitates, were dialyzed free of the sodium sulphate and concentrated as in the previous experiment. The precipitate from the 13.5% sodium sulphate fraction contained the antilysins; the filtrate of this fraction did not contain a trace of antilytic activity. The precipitates of all the remaining fractions were free of antilysins.

SUMMARY

Extracts in salt solution of alcohol precipitated bacteriophage show all the properties of the original bacteriophage; the alcohol precipitated bacteriophage can be regenerated; it is transmissible, and it produces the same antibodies.

Heat inactivated bacteriophage produces antibodies just as unheated bacteriophage does, showing in this way a "toxoid" alteration similar to toxins and lysins. This inactivated bacteriophage neutralizes antibacteriophagic serum, demonstrating the presence of a toxophore and haptophore group.

Antibacteriophagic bodies are salted out from the antiserum with the euglobulin fraction.

GENERAL INDEX

	A	PAGE
Abortin reaction of testicle	- - - - -	489
Abortion, infectious, bovine	- - - - -	498
Acrolein, formation of, from glycerol by <i>B. welchii</i>	- - - - -	282
<i>Actinomyces necrophorus</i>	- - - - -	390
<i>Aerobacter aerogenes</i> , destruction of uric acid by	- - - - -	479
Agglutination in infectious bovine abortion	- - - - -	498
Agglutination, surface energy in	- - - - -	5
Agglutinin formation, effect of suprarenalectomy on	- - - - -	334
Agglutinins, typhoid, permeability of rabbit placenta to	- - - - -	567
ALMQUIST, ERNST. Investigations on bacterial hybrids	- - - - -	341
Amboceptor pseudo-globulin, fractionation of	- - - - -	519
Anaerobic cultures, improved method for	- - - - -	581
ANDERSON, BELLE G. Gaseous metabolism of some anaerobic bacteria.		
XX. Experimental data	- - - - -	244
ANDERSON, BELLE G. Gaseous metabolism of some anaerobic bacteria.		
XIX. Methods	- - - - -	213
"Antigens, residue," of meningococci	- - - - -	537
Antitoxin, production of	- - - - -	549
ARNOLD, LLOYD, AND WEISS, EMIL. A study of bacteriophage with anti-bacteriophagic serum	- - - - -	505
ARNOLD, LLOYD, AND WEISS, EMIL. The Twort-d'Herelle phenomenon.		
The resemblance of bacteriophage to toxins and ferments	- - - - -	603
ARNOLD, LLOYD; SEE WEISS, E.	- - - - -	23
	B	
<i>B. botulinus</i> , estimations of growth of	- - - - -	105
<i>B. botulinus</i> in excretions	- - - - -	207
<i>B. botulinus</i> , inhibitive influence of sugars and salts on	- - - - -	134
<i>B. botulinus</i> , isolation of from feces	- - - - -	305
<i>B. botulinus</i> , optimum H-ion concentrations for	- - - - -	105
<i>B. botulinus</i> , resistance of spores of, to disinfectants	- - - - -	156
<i>B. botulinus</i> toxin, effects of light and heat on potency of	- - - - -	361
<i>B. botulinus</i> , type C., relationships of	- - - - -	347, 353
<i>B. diphtheriae</i> , bacteriophage for	- - - - -	401
<i>B. paratuberculosis</i> "Seddon"	- - - - -	347, 353
<i>B. pestis</i> in blood, bile and urine	- - - - -	291
<i>B. welchii</i> , formation of acrolein from glycerol by	- - - - -	282
<i>Bacillus acidophilus</i>	- - - - -	89
<i>Bacillus bifidus</i>	- - - - -	77
<i>Bacillus tertius</i> from stools	- - - - -	502
Bacteria, anaerobic and sporebearing, in human intestine	- - - - -	423
Bacteria, anaerobic, gaseous metabolism of	- - - - -	213, 244
Bacteria in middle ear infections	- - - - -	177
Bacteria, vitamins in growth of	- - - - -	311

	PAGE
Baeterial hybrids - - - - -	341
Baeterial metabolism - - - - - 67, 77, 89, 213,	244
Baeteriophage and antibaeteriophage serum - - - - -	505
Bacteriophage for <i>B. diphtheriae</i> - - - - -	401
Baeteriophage, resemblance of, to toxins and ferments - - - - -	603
BALDWIN, F. M. SEE WERKMAN, C. H. - - - - -	549
Bile as solvent for pneumococci - - - - -	327
BLAIR, JOHN E. A lytic principle (bacteriophage) for <i>Corynebacterium</i> diphtheriae - - - - -	401
Blood, Rocky Mountain spotted fever in - - - - -	587
Botulinum, Cl., effect of Cl. sporogenes on - - - - -	576
Botulinus toxin, effects of light and heat on potency of - - - - -	361

C

Chaneroidal infection - - - - -	591
Cl. botulinum, effect of Cl. sporogenes on - - - - -	576
Cl. sporogenes, effect of, on Cl. botulinum - - - - -	576
Colon-aerogenes group, tests for in water - - - - -	14
Colon-aerogenes group utilization of citrate by - - - - -	315
CONNOR, CHARLES L. The identification of the organism of Rocky Mountain spotted fever in the blood - - - - -	587
Cultures, anaerobic, improved method for - - - - -	581

D

DACK, GAIL M.; SEE JORDAN, EDWIN O. - - - - -	576
Diphtheria, myocardial lesions of - - - - -	32
Diphtheria toxin, resistance of rat to - - - - -	549
DOZIER, CARRIE CASTLE. Inhibitive influence of sugars and salt on viability, growth, and toxin production of <i>B. botulinus</i> . XVII - - - - -	134
DOZIER, CARRIE CASTLE. Optimum and limiting hydrogen-ion concentrations for <i>B. botulinus</i> and quantitative estimations of its growth. XVI - - - - -	105
DOZIER, CARRIE CASTLE. Resistance of spores of <i>B. botulinus</i> to disin- fectants. XVIII - - - - -	156

E

EASTON, E. J. AND MEYER, K. F. Occurrence of <i>Bacillus botulinus</i> in human and animal excretion. XXI - - - - -	207
EBERTZ, EDWIN G. SEE ROCKWELL, GEORGE E. - - - - -	573
ECKER, E. E., AND MORRIS, J. LUCIEN. Factors influencing the destruction of uric acid by <i>Aerobacter aerogenes</i> - - - - -	579
ECKER, E. E.; SEE MEGRAIL, E. - - - - -	1
Electrodialysis, fractionation of pseudo-globulin by - - - - -	519
Enteritidis-paratyphoid B. group in guinea-pigs - - - - -	407

F

Ferments, resemblance of bacteriophage to - - - - -	603
Fever, Rocky Mountain spotted - - - - -	587
Flocculation test for syphilis - - - - -	540

G

PAGE

Glycerol, formation of acrolein from - - - - -	282
GREEN, R. G., AND HALVORSON, H. O. Surface energy as the controlling factor in agglutination and dispersion - - - - -	5
GUSSIN, HARRY; SEE KELLY, F. B. - - - - -	323, 327
GUYER, M. F., AND SMITH, E. A. Permeability of the rabbit placenta to precipitins and to typhoid agglutinins - - - - -	567

H

HAGAN, WILLIAM A. Formation of peroxide by actinomyces necrophorus on exposure to air in relation to anaerobic plate cultures - -	390
HALL, IVAN C., AND MATSUMURA, KIYOSHI. Recovery of <i>Bacillus tertius</i> from stools of infants - - - - -	502
HALVORSON, H. O.; SEE GREEN, R. G. - - - - -	5
HANER, REBA CORDELIA; SEE KENDALL, A. I. - - - - -	67, 77, 89
Heart lesions in diphtheria - - - - -	32
HEKTOEN, LUDVIG, AND WELKER, WILLIAM H. Precipitin reactions of serum proteins - - - - -	295
HIRSCH, EDWIN F.; SEE LOCKE, ARTHUR - - - - -	519
HUMPHREYS, ELEANOR M.; SEE WHEELER, MARY W. - - - - -	305
HUMPHREYS, FREDERICK B. Formation of acrolein from glycerol by <i>B. welchii</i> - - - - -	282

I

Infection, chancroidal - - - - -	591
Infections of middle ear - - - - -	177

J

JAFFE, HENRY L., AND MARINE, DAVID. Effect of suprarenalectomy in rats on agglutinin formation - - - - -	334
JORDAN, EDWIN O., AND DACK, GAIL M. The effect of <i>Cl. sporogenes</i> on <i>Cl. botulinum</i> - - - - -	576

K

KAHN, MORTON CHARLES. Anaerobic spore-bearing bacteria of the human intestine in health and in certain diseases - - - - -	423
KELLY, FRANK B., AND GUSSIN, HARRY. Studies on respiratory diseases. XIX. Untreated bile as a solvent for pneumococci - - - - -	327
KELLY, FRANK B., AND GUSSIN, HARRY. Studies on respiratory diseases. XVIII. The relative reliability of throat swabs for isolating the causative pneumococcus type - - - - -	323
KENDALL, ARTHUR ISAAC, AND HANER, REBA CORDELIA. <i>Bacillus acidophilus</i> . LXXII. Studies in bacterial metabolism - - - - -	89
KENDALL, ARTHUR ISAAC, AND HANER, REBA CORDELIA. <i>Bacillus bifidus</i> . LXXI. Studies in bacterial metabolism - - - - -	77
KENDALL, ARTHUR ISAAC, AND HANER, REBA CORDELIA. <i>Micrococcus ovalis</i> . LXX. Studies in bacterial metabolism - - - - -	77
KOSER, STEWART A. Differential tests for colon-aerogenes group in relation to sanitary quality of water - - - - -	14
KOSER, STEWART A. Is ability to utilize citrate readily acquired or lost by the colon-aerogenes group? - - - - -	315

	PAGE
L	
LOCKE, ARTHUR, AND HIRSCH, EDWIN F. The isolation of substances with immune properties. I. The fractionation of iso-electric amboceptor pseudo-globulin by electro-dialysis - - - - -	519
M	
MARINE, DAVID; SEE JAFFE, HENRY L. - - - - -	334
MARSHALL, M. S. Surface tension of culture mediums - - - - -	526
MATHEWS, FRANK P. A Study of the agglutination test for bovine infectious abortion - - - - -	498
MATSUMURA, K.; SEE HALL, I. C. - - - - -	502
MCGLUMPHY, C. B. Practical results with a flocculation test for syphilis -	540
Mediums, culture, surface tension of - - - - -	526
MEGRAIL, EMERSON, AND ECKER, E. E. Stability of pneumococcus types in sterile abscesses - - - - -	1
Meningococci, specific "residue antigens" of - - - - -	537
Metabolism bacterial - - - - - 67, 77, 89, 213,	244
MEYER, K. F.; SEE GASTON - - - - -	207
MEYER, K. F.; SEE SCHOENHOLZ, P. - - - - -	361
Micrococcus ovalis - - - - -	67
MORRIS, J. LUCIEN; SEE ECKER, E. E. - - - - -	479
Myocardial lesions in diphtheria - - - - -	32
N	
NELSON, V. E.; SEE WERKMAN, C. H. - - - - -	549
O	
OHOTO, OSAMU. B. pestis in blood, bile and urine - - - - -	291
Organism of Rocky Mountain spotted fever - - - - -	587
Otitis media - - - - -	177
P	
Permeability of rabbit placenta - - - - -	567
Peroxide, formation of by Actinomyces necrophorus - - - - -	390
PFENNINGER, W. Toxic-immunologic and serologic relationship of B. botulinus, type C, and B. parobotulinus. "Seddon." XXII - -	347
Phenomenon, Twort-d'Herelle - - - - -	603
Placenta, rabbit, permeability of - - - - -	567
Pneumococci, bile solubility of - - - - -	327
Pneumococcus, stability of types in abscesses - - - - -	1
Pneumococcus types, isolation of, from throat swabs - - - - -	323
Precipitins for serum proteins - - - - -	295
Precipitins, permeability of rabbit placenta to - - - - -	567
Properties, germicidal, of chemically pure soaps - - - - -	557
Proteins, serum, precipitin reactions of - - - - -	295
PRZESMYCKI, FELIX. Specific "residue antigens" of different types of meningococci - - - - -	537
Pseudo-globulin, fractionation of - - - - -	519

R

	PAGE
Rat, resistance of, to diphtheria toxin - - - - -	549
Respiratory infections - - - - -	323, 327
ROBERTSON, R. C. Food accessory factors (vitamines) in bacterial growth. IX. Growth of several common bacteria in a synthetic medium and relation of substances formed by them to growth of yeast - - -	311
ROCKWELL, GEORGE E. An improved method for anaerobic cultures -	581
ROCKWELL, GEORGE E., AND EBERTZ, EDWIN G. How salt preserves -	573
Rocky Mountain spotted fever - - - - -	587

S

SAELHOF, CLARENCE C. Observations on chancroidal infection - -	591
Salt, how it preserves - - - - -	573
SCHOENHOLZ, P., AND MEYER, K. F. Effect of direct sunlight, diffuse day- light and heat on potency of botulinus toxin in culture mediums and vegetable products. XXIV - - - - -	361
Serum proteins, precipitin reactions of - - - - -	295
SEYFARTH, MAC HARPER. The abortin reaction in the testicle as an indi- cator of the hypersensitiveness of infection - - - - -	489
SMITH, E. A.; SEE GUYER, M. F. - - - - -	567
Soaps, chemically pure, germicidal properties of - - - - -	557
Sporogenes, Cl., effect of, on Cl. botulinum - - - - -	576
Substances with immune properties, isolation of - - - - -	519
Suprarenalectomy, effect of, on agglutinin formation - - - - -	334
Surface energy in agglutination and dispersion - - - - -	5
Syphilis, flocculation test for - - - - -	540

T

Tension, surface, of culture mediums - - - - -	526
Testicle, abortin reaction of - - - - -	489
Test, flocculation, for syphilis - - - - -	540
THOMAS, BERNARD G. H. Occurrence of organisms of the enteritidis para- typhoid B group in guinea-pigs - - - - -	407
Toxin, diphtheria, resistance of rat to - - - - -	549
Toxins, resemblance of bacteriophage to - - - - -	603
Twort-d'Herelle phenomenon - - - - -	603
Typhoid agglutinins, permeability of rabbit placenta to - - - - -	567

U

Uric acid, destruction by <i>Aerobacter aerogenes</i> - - - - -	479
---	-----

V

VALENTINE, EUGENIA. Bacteriologic study of middle ear infections - -	177
Vitamins in bacterial growth - - - - -	311
Vitamins, immunologic significance of - - - - -	549

W

WAGNER, E. Biochemical activities of <i>B. botulinus</i> , type C, and B. Para- botulinus, "Seddon." XXIII - - - - -	353
WALKER, JOHN E. The germicidal properties of chemically pure soaps -	557

	PAGE
WARTHIN, ALDRED SCOTT. The myocardial lesions of diphtheria - -	32
Wassermann antigens - - - - -	23
Water, tests for colon-aerogenes group in - - - - -	14
WEISS, EMIL, AND ARNOLD, LLOYD. Specific and nonspecific properties of Wassermann antigens - - - - -	23
WEISS, EMIL; SEE ARNOLD, LLOYD - - - - -	505, 603
WELKER, WM. H.; SEE HEKTOEN, LUDVIG - - - - -	295
WERKMAN, C. H., BALDWIN, F. M., AND NELSON, V. E. Immunologic sig- nificance of vitamins. V. Resistance of the avitaminic albino rat to diphtheria toxin; production of antitoxin and blood pressure effects -	549
WHEELER, MARY W., AND HUMPHREYS, ELEANOR M. Isolation of B. botu- linus, type B, from feces by use of blood agar plates in anaerobic jar -	305

Y

Yeast, bacterial vitamins in growth of - - - - -	305
--	-----

402

